

A STUDY OF NON-CULTURAL METHODS FOR DETECTING
INFECTION WITH NEISSERIA GONORRHOEAE

by

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B.Sc. Honours

Thesis presented for the Degree of Doctor of Philosophy
at the University of Edinburgh in the Faculty of Medicine

1981



To my parents in appreciation of their support
provided at a time of great hardship and sacrifice.

This is to certify that the material presented here is the candidate's own work.

The results of some of these studies have been published jointly with other research colleagues; the relevant references are:

Sarafian, S.K. and Young, H. 1980. Identification of pathogenic neisseriae by genetic transformation. Journal of Medical Microbiology, 13, 291-296.

Young, H., Sarafian, S.K., and McMillan, A. 1981. Reactivity of the Limulus lysate assay with uterine cervical secretions: a preliminary evaluation. British Journal of Venereal Diseases, 57, 200-203.

Some of the results were communicated at a poster session at the Workshop on the Genetics and Immunobiology of Pathogenic Neisseria, held in Hemavan, Sweden, June 1980 (see Appendix A).

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ACKNOWLEDGEMENTS

I would like to express my gratitude to Dr Hugh Young for his patient supervision of the project, and to Professor J.G. Collee for his support and useful criticism of the manuscript. I am also most grateful to Dr D.M. Weir for his general advice and intellectual stimulation. I am also indebted to the Faculty of Medicine for granting me a two-year scholarship.

I wish to thank Dr A. McMillan who has been an invaluable link with the Department of Genito-Urinary Medicine, and Dr D.H.H. Robertson, Head of the University Department of Genito-Urinary Medicine, for his cooperation.

My thanks also go to:

My colleagues in the Research and Development Laboratory for their support.

The diagnostic section of the Department of Bacteriology, and especially to Mr A.B. Harris for his kind help with the identification of bacterial species.

Dr I. Poxton and Mr R. Brown for invaluable advice on qualitative and quantitative analytical procedures.

The Virus Diagnostic Laboratory for identifying isolates of Chlamydia trachomatis.

Mrs E. Ross and Mrs A. Moffat for their patient advice and assistance with preparation of media.

Mr W. Marr and Mr D. Brown for practical advice.

The staff in the Animal House for help with raising antisera.

The Audio-Visual Centre for the preparation of the art work.

Mrs M. Cole, Mrs L. Bryson and Mrs J. Collins for their kind secretarial assistance.

Mrs V. McGrath for the preparation of the typescript.

I wish to thank warmly my friends Kamal, Marianne, Andréa, Moira and Amal, and especially my tante Alice for reasons she knows best.

SUMMARY

Because of the short incubation period of gonorrhoea and the high infectivity of the disease, rapid and accurate diagnosis followed by immediate and effective treatment are essential for the control of infection within a community.

Most of the methods available for the laboratory diagnosis of gonorrhoea are based on culture of the organism and depend on maintaining the viability of N. gonorrhoeae. The aim of this study was to investigate an alternative approach to the diagnosis of gonorrhoea based on the detection of gonococcal components in secretions obtained from infected patients.

The methods investigated comprised: genetic transformation, enzyme-linked immunosorbent assay, coagglutination, and detection of gonococcal endotoxin by the Limulus lysate assay. In parallel with the development of these tests, qualitative and quantitative assessments of the microbial content of cervical and vaginal aspirates were made. There was a wide variation in the numbers of gonococci aspirated in cervical secretions; the mean number was 1.02×10^6 c.f.u./ml, and the values ranged from 5.4×10^3 to 8.0×10^6 c.f.u./ml.

The detection of N. gonorrhoeae by genetic transformation of a naturally occurring proline auxotroph of N. gonorrhoeae strain F62 is described. Of 169 clinical isolates of N. gonorrhoeae, approximately 90 per cent gave positive results in the transformation assay. Of the other neisseriae tested, only N. lactamica and meningococci gave a positive transformation assay; however, the sensitivity of the assay was found to be approximately 1000-fold lower with

N. meningitidis as test organism. Although proline requirement did not appear to limit the value of the assay greatly, it was probably the main reason for negative results. Urethral swab specimens from 14 (36.8 per cent) of 38 infected men and cervical swab specimens from six (33.3 per cent) of 18 infected women gave a positive transformation result. With aspirates as test specimens, 15 (75 per cent) of 20 infected women were detected by the transformation assay.

An indirect sandwich ELISA system was developed to detect gonococcal antigens. The feasibility of using rabbit antiserum raised against N. gonorrhoeae strain 9 to detect gonococcal LPS, whole cells, and outer membrane (OM) protein was investigated. OM protein was found to be the main antigen detectable with the antiserum. A positive assay result could be obtained with a minimum of 46 to 92 ng gonococcal OM protein, and/or with 6.6×10^3 c.f.u. of N. gonorrhoeae. The assay detected 12 (60 per cent) of 20 infected women. There was no correlation between viable counts for N. gonorrhoeae and the assay results, and it was concluded that the assay depended largely on the availability of soluble antigen. Cervical aspirates from two (11.7 per cent) of 17 culture-negative patients gave a false-positive result.

A preliminary evaluation of the Limulus lysate assay to detect gonococcal endotoxin was carried out with cervical secretions tested at a dilution of 1 in 100. The assay detected up to 17 (70.8 per cent) of 24 infected women, when those secretions giving a negative result were re-tested at a dilution of 1 in 50. The false-positive rate was 7.3 per cent. In a second trial with

bacteriologically investigated cervical aspirates, the assay detected up to 18 (94.7 per cent) of 20 infected patients. The presence of other Gram-negative organisms in the aspirates of culture-negative patients did not interfere with the assay.

The coagglutination test was not sensitive enough for detecting gonococcal components in cervical secretions. The transformation assay, with an auxotrophic indicator strain, is too laborious and technically demanding for use on a routine basis. With further developments, the Limulus lysate assay and the ELISA system might make significant contributions to the laboratory diagnosis of gonorrhoea.

GENERAL INTRODUCTION

INTRODUCTION

Gonorrhoea, predominantly an infection of the mucosal surfaces of the genito-urinary tract with the bacterium Neisseria gonorrhoeae, is mainly transmitted by sexual intercourse. Allusions in biblical writings substantiate the existence of gonorrhoea since antiquity (Morton, 1977). The disease was known to the Chinese Emperor Huang-ti (2637 BC) who defined it as an inflammation of the urethra caused by contact of the penis with matter of a peculiar nature emitted by the genital organs of the woman (Fiumara, 1972). The first scientific observations on the disease are attributed to Hippocrates, the "Father of Medicine" (460-355 BC). He called it "strangury" and was in no doubt that it "resulted from indulgence in the pleasures of Venus". It was Galen (130-200 AD) who introduced the term "gonorrhoea" which means literally a "flow of seed". The first description of a contagious urethritis recognizable as the gonorrhoea of today is recorded in a manuscript in 1376 by John of Arderne, surgeon to Richard II and Henry IV. In 1879, long after Anthony van Leeuwenhoek had discovered the world of "animalcules", the aetiology of the infection was clearly established when Albert Neisser gave the first detailed and accurate description of the causative organism in exudate. Bumm, in 1885, grew the organism on artificial medium and showed it to cause gonorrhoea in human volunteers. In 1884, Hans Gram described his method of staining bacteria; Gram's stain, or one of its modifications, is now widely used for the presumptive diagnosis of gonorrhoea (Thayer and Moore, 1964; Morton, 1977).

At present, gonorrhoea is one of the most commonly reported infectious diseases in many areas of the world. The true incidence of gonorrhoea is difficult to determine and under-reporting is widespread. In the U.S.A. alone, three million cases are thought to occur annually, and the disease is obviously out of control (Catterall, 1981). Paradoxically, the infection has flourished in the antibiotic era. The late 1930s witnessed the first effective therapy of gonorrhoea with the sulphonamides replacing treatment by local washes and by weak antiseptics taken orally and excreted in the urine. With the development of resistance, the decreased efficacy of the sulphonamides soon disappointed (Dunlop, 1949). With the advent of penicillin therapy and the more settled conditions of peacetime, there was a marked decline in the incidence of gonorrhoea between the mid 1940s and mid 1950s. Unfortunately the trend soon reversed and a progressive increase in incidence was noted: in 1978 a total of 57 501 cases of gonorrhoea were reported in the clinics of England (Extract from the Annual Report of the Chief Medical Officer of the Department of Health and Social Security, 1980) compared with the wartime peak of 47 343 in England and Wales (Fig. 1).

One of the problems in controlling gonorrhoea is the short incubation period, usually 2 to 8 days. In men, the disease usually presents clinically as a purulent urethral discharge combined with dysuria of varying severity. In women, the infection is often asymptomatic and may produce cervicitis, urethritis, and bartholinitis. In a proportion of patients the infection may involve the upper genital

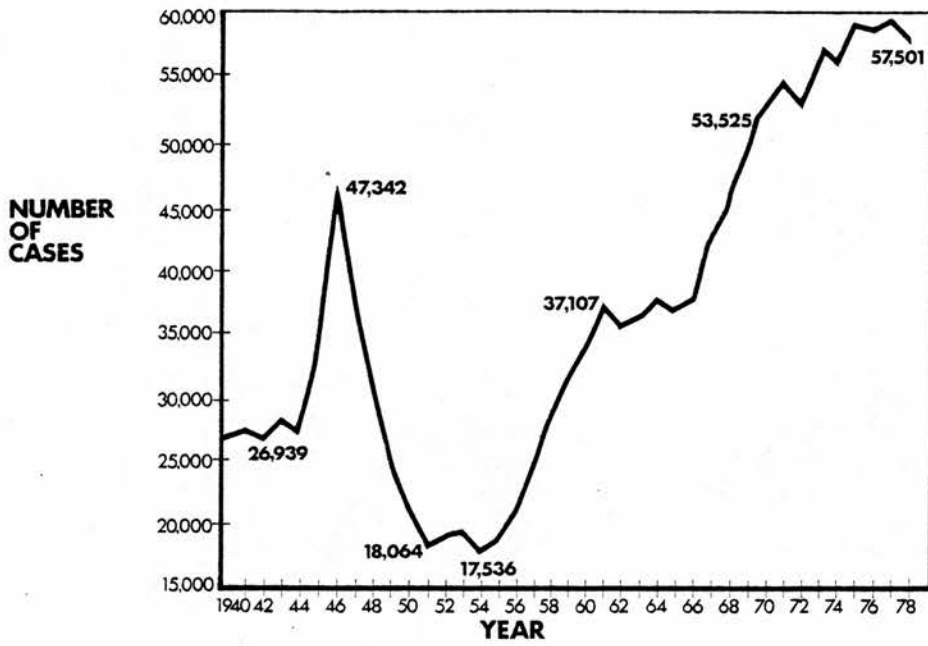


Fig. 1 Number of cases of gonorrhoea reported annually in England and Wales from 1940-68 and in England alone from 1969-78.

tract, and in some instances a disseminated gonococcal infection may arise (Handsfield, 1978; Robertson, McMillan and Young, 1980).

Other problems associated with the control of gonorrhoea have been discussed by Willcox (1981). He considers altered mores and behaviour, emancipation of women, male homosexuality, population mobility, and developing resistance of the gonococcus to be among the most important factors.

It has become increasingly clear that antibiotics and chemotherapeutic agents are not in themselves the ultimate solution to the problem. Growing resistance to antibiotics and the recent appearance of beta-lactamase producing strains of N. gonorrhoeae present a more ominous picture for the future. Health education and the wider dissemination of factual information to the public has recently been favoured by many health authorities, and modern methods of education have been used to these ends. Unfortunately the available evidence suggests that knowledge of the facts does not necessarily influence human behaviour (Catterall, 1976).

Until or unless immunoprophylaxis can be shown to be both feasible and effective, the basic approaches to the control of gonorrhoea must be improved. These include the provision of an adequate network of public clinics offering prompt, scientifically based diagnosis and treatment, the tracing and treatment of all infected persons, and public and professional education concerning all aspects of gonorrhoea. Because of the short incubation period of gonorrhoea and high infectivity, rapid and accurate diagnosis followed by immediate treatment are essential for the control of

infection within the community. The consensus of informed opinion suggests that no one diagnostic test is one hundred per cent reliable, and it is therefore common practice to establish the diagnosis by one or more of a variety of methods. The development of a reliable, rapid and cheap diagnostic test and its widespread application could be of great value in the control of the disease.

The purpose of the following introduction is to review relevant aspects of the biology of the gonococcus, host-parasite interactions, and laboratory procedures for the diagnosis of gonococcal infection.

THE BIOLOGY OF NEISSERIA GONORRHOEAE

Gonococci are small Gram-negative, kidney-shaped diplococci with the long axes parallel and the opposed surfaces slightly concave. By microscopy, it is impossible to differentiate the gonococcus from Neisseria meningitidis or from other non-pathogenic or potentially pathogenic neisseriae commonly found in the upper respiratory tract, and occasionally on the mucous surfaces of the genito-urinary tract, particularly in the female (Griffis and Artenstein, 1976).

In 1963 Kellogg and his co-workers described four distinct colony-types of N. gonorrhoeae referred to as T1 to T4, and demonstrated the predominance of colony-type 1 in primary culture from clinical material. Later, two additional colony-types were recognized: T5 (Jephcott and Reyn, 1971) and T1¹ (Chan and Wiseman, 1975). On the basis of studies on the virulence of the different colony-types in human volunteers (Kellogg, 1963) and the chick embryo (Chan and Wiseman, 1975) it is recognized that colony-types 1, 2, and 1¹ are the virulent clinically-associated types, whereas colony-types 3, 4, and 5 are the essentially avirulent laboratory-associated types. Colony-types 1, 2, and 1¹ can be maintained in vitro by selective subculture at daily intervals; non-selective subculture leads to the emergence of colony-types 3, 4, and 5. The presence of antimicrobial agents in the growth medium causes profound changes in colonial morphology and makes colony-typing difficult (James, Wende and Williams, 1973). Chan and Wiseman (1975) reported that the typical patterns for colonial variation

that they observed were T1 to T4, T2 to T3, T3 to T4, and T1¹ to T5. When N. gonorrhoeae is propagated on a clear medium, colonies with markedly differing colour and opacity characteristics are found (Swanson, 1978a).

Gonococci are antigenically heterogeneous and no widely accepted method of typing strains has yet been developed. This represents a major handicap in the study of the epidemiology and immunology of the disease. Consequently, the immunochemical characterization of the various cell surface components of N. gonorrhoeae has received much attention.

1. The cell envelope

The gonococcal cell surface conforms to the general characteristics of the envelopes of Gram-negative bacteria. It consists of an undulating outer membrane structure, an intermediate layer of peptidoglycan, and an inner (plasma or cytoplasmic) membrane (Fitz-James, 1964; Swanson, Kraus and Gotschlich, 1971).

An electron microscopic examination to compare gonococci present in urethral exudate with those grown in vitro revealed distinct differences in the appearance of their cell surface profiles (Novotny, Short and Walker, 1975). Gonococci in urethral exudate presented a smooth appearance with parallel surface layers and a thick peptidoglycan layer; they also possessed a "fuzzy" coat external to the outer membrane. This structure was absent in cells maintained in the laboratory; these were rough and their surface layers appeared to be disorganized. The same observation concerning the prominent peptidoglycan layer of animal-adapted gonococci compared with cells grown in vitro was made by Arko,

Bullard and Duncan (1976).

The outer membrane of gonococci possesses pit-like structures which can be seen in negatively stained preparations (Swanson et al., 1971; Novotny et al., 1975). Another unusual feature of N. gonorrhoeae is the membrane-bound vesicles found in the cytoplasm of some gonococci (Murray, Reyn and Birch-Andersen, 1963; Fitz-James, 1964). Until additional evidence is presented, the possibility of these structures being artefacts produced during the fixation and/or embedding process cannot be excluded.

1.1 Pili

Pili can be defined as rod-like non-flagellar bacterial appendages assembled from protein subunits (Brinton, 1959; Brinton, Gemski and Carnahan, 1964). They can be divided into two broad classes: somatic or type 1 pili and conjugal or sex pili. Whereas both provide specific sites of attachment for a variety of bacteriophages, the latter mediate the transfer of deoxyribonucleic acid by conjugation while somatic pili are involved in functions such as adhesion (Swaney et al., 1977).

Pili were first reported on gonococci by Jephcott, Reyn and Birch-Andersen in 1971, and independently by Swanson et al. (1971). These structures, with a diameter of about 7 nm (Robertson, Vincent and Ward, 1977), were seen to extend from the surface of gonococci from the virulent colony-types 1 and 2 but not from the avirulent types 3 and 4 (Jephcott et al., 1971). Novotny and Turner (1975) reported that the serum from rabbits immunized with a suspension of type 3 organisms (with no detectable pili) contained antibodies that reacted with pili of the homologous type 2 strain. They

proposed that the pilus antigen in type 3 gonococci may exist in a form different from the typical gonococcal pili seen in type 1 and 2 cells.

Pili have also been demonstrated on non-pathogenic neisseriae (Wistreich and Baker, 1971; McGee et al., 1977) and on N. meningitidis (Devoe and Gilchrist, 1974; McGee et al., 1977). Short pili (175-210 nm in length) are seen only on non-pathogenic neisseriae, whereas long pili (up to 4300 nm) are seen on pathogenic as well as non-pathogenic neisseriae (McGee et al., 1977). Colonies of N. meningitidis and N. pharyngis which consisted of piliate organisms showed no difference from those colonies consisting of organisms that lacked pili. On the other hand, morphologically different colony-types of N. subflava were formed by piliate organisms that were indistinguishable by electron microscopy (McGee et al., 1977). Furthermore, gonococcal colonies of types 1 and 2 have different morphologies, even though both consist of piliate organisms with the same morphological characteristics (Jephcott et al., 1971; Swanson et al., 1971). These observations suggest that pili either have no influence on, or are not the only factor responsible for the morphology of colonies of virulent gonococci.

Much attention has been focused on gonococcal pili in view of their possible role in a first critical step of attachment to epithelial cells in human infection (Punsalang and Sawyer, 1973; Buchanan and Pearce, 1976); the significance of pili in the host-parasite relationship is discussed later. Pili have been used in vitro as antigen in serological tests (Buchanan et al., 1973; Reimann and Lind, 1977), and also their use as antigen in a vaccine

has been investigated (Brinton et al., 1978).

Gonococcal pili were first purified by Buchanan and co-workers (Buchanan, Swanson and Gotschlich, 1972; Buchanan et al., 1973; Buchanan, 1975). The molecular weight of the pilus subunit, pilin, is approximately 18 000 to 20 000 daltons and varies somewhat in different strains of N. gonorrhoeae (Buchanan and Pearce, 1976; Robertson et al., 1977; Salit, Blake and Gotschlich, 1980). Heterogeneity of gonococcal pili has also been demonstrated by immunological methods (Buchanan, 1975; Novotny and Turner, 1975).

In addition to inter-strain differences, there are demonstrable intra-strain differences in the isoelectric point and buoyant density of pili derived from opacity variants. Lambden, Robertson and Watt (1980) demonstrated the presence of two distinct types of pili, designated alpha and beta, produced by isogenic variants of N. gonorrhoeae P9. The transparent variant produced alpha-pili and the intermediate/opaque variant produced beta-pili; the molecular weights of their pilin subunits were 19 500 and 20 500 respectively. In addition, preparations of radioiodinated alpha- and beta-pili were tested for their ability to attach to human buccal epithelial cells (Lambden et al., 1980). Striking differences between alpha- and beta-pili were found when attachment was measured over a broad pH range. The attachment of alpha-pili was markedly pH dependent with a maximum binding of 44 per cent at pH 6.5. By contrast, beta-pili showed a steady decline in binding ability over the pH range tested for a maximum attachment of 13 per cent at pH 4.5 to 4 per cent at pH 8.5. Moreover, removal of sialic acid residues from buccal epithelial cell surface carbohydrates by

neuraminidase treatment markedly inhibited the binding of alpha-pili, but had little effect on beta-pili. Further treatment of the neuraminidase-modified buccal epithelial cells with a mixture of exoglycosidases reduced the binding of alpha-pili to a level comparable to that of beta-pili. Lambden et al. (1980) suggested that a possible explanation is that alpha-pili specifically bind to a receptor, involving sialic acid plus other sugar residues, present on the surface of human buccal epithelial cells and that beta-pili lack such receptor recognition. Furthermore, binding to this receptor is pH dependent, with an optimum at pH 6.5.

The amino acid composition of purified pili from strains P9 and 201 of N. gonorrhoeae was reported by Robertson et al. (1977). In both strains the pilin subunit had approximately the same number of amino acid residues, 208 in strain P9 and 212 in strain 201, giving calculated molecular weights of 22 600 for P9 and 22 352 for 201. The molar ratios of the amino acids for the two strains were virtually identical with the exception of proline. Non-polar amino acids comprise approximately 46 per cent of the pilin subunits of strains P9 and 201 gonococcal pili (Robertson et al., 1977). Hermodson, Chen and Buchanan (1978) demonstrated that gonococcal pilin has an unusual amino-terminal amino acid, N-methylphenylalanine, and also confirmed the hydrophobic nature of the protein: the first 24 amino acid residues are predominantly hydrophobic with two hydrophilic residues only, corresponding to threonine and glutamic acid at positions 2 and 5 respectively.

No amino sugars were detected in a hydrolysate of purified P9 pili using a specially programmed amino acid analyser (Robertson et al., 1977). Additional components were detected in pili from

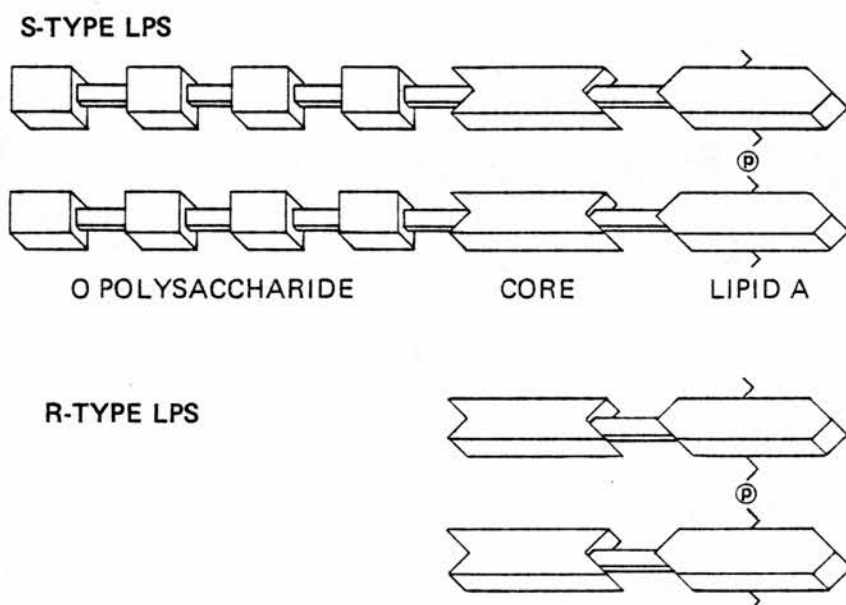
strain P9. Neutral sugars comprised approximately 1.3 per cent (w/w) of the pili. Gas chromatographic analysis revealed the presence of galactose and also trace amounts of glucose. No heptose or pentose was detected, indicating the absence of lipopolysaccharide or nucleic acids. Phosphate analysis of purified P9 pili indicated the presence of 1 to 2 phosphate groups per pilin subunit. Robertson et al. (1977) suggested that the phosphate would alter the overall charge, thereby increasing the electrophoretic mobility of the pilin subunit. This would explain why molecular weights estimated from SDS-polyacrylamide gel electrophoresis were lower than those calculated from the amino acid analyses. The phosphate groups may be functional; Brinton (1971) has suggested that the phosphate present in Escherichia coli F pilus subunit may provide energy for the polymerization of pilin subunits to form the pilus strand, or to orientate the subunits correctly for pilus synthesis.

1.2 Lipopolysaccharide

Extensive physical and chemical investigations have been made on the lipopolysaccharides (LPS) isolated from the outer membrane of enterobacteria (Lüderitz, Jann and Wheat, 1968). Lipopolysaccharides are long-chain phosphorus-containing heteropolymers consisting of lipid A covalently linked through 2-keto-3-deoxy-octulosonic acid (KDO) to a common core polysaccharide; to this is attached a high molecular weight polysaccharide made up of species-specific identical oligosaccharide repeating units (Lüderitz et al., 1968; Perry, Diena and Ashton, 1977). Lipopolysaccharides are also known as endotoxins because of their pyrogenic and toxic

effects in animals (Lüderitz et al., 1968).

The species-specific polysaccharide side-chains extend far out from the cell surface (Shands, 1965) and are known as somatic or O-antigens. The LPS described above is referred to as S-type (smooth) LPS, and LPS lacking the O chain (composed of only core oligosaccharide and lipid A) is referred to as R-type (rough) LPS. The two types of LPS can be represented diagrammatically as follows:



R-type LPS arises in cells defective in the synthesis of O polysaccharide. However defects in the synthesis of the core itself also result in the production of R-type LPS, because the O polysaccharide, though synthesized, cannot be transferred to the incomplete core which lacks the necessary acceptor site (Lüderitz et al., 1968).

LPS is apparently located on the outermost aspect of the outer membrane of the Gram-negative cell envelope (Shands, 1965; Shands,

1966; Mergenhagen, Bladen and Hsu, 1966). This is also suggested by the ease with which mild treatment can result in LPS release (Leive, Shovlin and Mergenhagen, 1968). Spontaneous release of LPS from Gram-negative bacteria has also been documented (Crutchley, Marsh and Cameron, 1968; Rothfield and Pearlman-Kothencz, 1969). The production of free endotoxin seems to be a general phenomenon observable in all Gram-negative bacteria, and its production has been visualized as a process of "blebbing" of outer membrane (Devoe and Gilchrist, 1973). Free LPS has also been found in culture supernates of N. meningitidis (Zollinger et al., 1972; Devoe and Gilchrist, 1973), and a number of other Neisseria species (Johnson et al., 1975; Johnson, McDonald and Perry, 1976; Johnson, Perry and McDonald, 1976); the free LPS isolated from the culture medium was found to be identical with the corresponding cell-bound LPS (Zollinger et al., 1972).

Stead et al. (1975) observed long projections extending from the outer membrane of gonococci. These projections presumably contained LPS and had a tendency to break off into vesicles. Present evidence suggests that LPS is located in the outer membrane of N. gonorrhoeae as found in other Gram-negative bacteria (Johnston and Gotschlich, 1974; Wolf-Watz et al., 1975). Of the methods that have been applied to the extraction of LPS (Boor and Miller, 1944; Glynn and Ward, 1970; Maeland and Kristoffersen, 1971), extraction with aqueous phenol has proved popular because of its relative simplicity and also because it is one of the few methods by which LPS may be extracted from R-type bacteria (Johnson and Perry, 1976). Greater yields of LPS were obtained when cells were disrupted by treatment with lysozyme in the presence of ethylenediaminetetraacetic

acid (EDTA), or by grinding with glass beads prior to conventional phenol extraction (Johnson and Perry, 1976).

Stead et al. (1975) used a phenol water extraction procedure to prepare LPS from five strains of N. gonorrhoeae. Analyses showed that all the LPS preparations contained glucose, galactose, glucosamine, heptose, KDO, phosphate, and the same fatty acids. No significant differences were detected between the LPS preparations of gonococci from colony-types 1 and 4, or penicillin-sensitive and resistant strains. These gonococcal lipopolysaccharides appeared to lack O-antigen side chains.

In a similar type of study, Perry et al. (1975) examined the LPS from N. gonorrhoeae colony-types 1 and 4. Common to both were lipid A and a core oligosaccharide composed of 2-amino-2-deoxy-D-glucose, D-glucose, D-galactose, L-glycero-D-manno-heptose and KDO. The LPS preparations from colony-type 1 cells were characterized by giving high molecular weight O polysaccharides which showed considerable strain variation in glycosyl composition. These investigators proposed that colony-type 4 cells produced a common R-type LPS, whereas colony-type 1 cells produced an S-type LPS with structurally different O polysaccharide side chains.

Following the conflicting information obtained in these studies (Stead et al., 1975; Perry et al., 1975), Wiseman and Caird (1977) examined the LPS composition of 38 clinical isolates of N. gonorrhoeae, including isogenic strains of the five recognized colony-types. The concentrations of glucose, galactose, and mannose varied from strain to strain, but cells from colony-types 1 and 2 contained a greater total glycosyl concentration than cells from colony-types 3 to 5.

The higher mannose-KDO or galactose-KDO ratios in LPS from colony-types 1 and 2 suggests that these sugars are part of an O-specific chain, or at least that their concentration in cells of these colony-types is greater. In contrast to the findings of Perry et al. (1975), a uniform composition of glycoses was found in LPS from colony-type 1 cells together with variation in their concentration (Wiseman and Caird, 1977). No qualitative differences were observed between LPS from colony-types 1 and 4 gonococci apart from the presence of rhamnose in the latter; Perry et al. (1975) found rhamnose only in LPS from colony-type 1 gonococci.

The reasons for the differences between the data of Wiseman and Caird (1977) and those of Perry et al. (1975) are not immediately evident. Moreover, not consistent with either of these studies based on physicochemical methods was the demonstration by Apicella and Gagliardi (1979) that the serogroup (O-specific) antigen can be detected in LPS polysaccharides isolated from colony-type 4 gonococci, when preparations are studied immunologically. Differences in culture media and growth conditions may be partly responsible. McDonald and Adams (1971) observed that environmental conditions affected the content and composition of the LPS in N. sicca; LPS from organisms grown at a high aeration rate (high growth rate) had a greater content of hexosamine and KDO, and a higher galactosamine to glucosamine ratio than LPS from organisms grown at a low aeration rate (low growth rate).

Evidence of the common antigenic nature of gonococcal LPS comes from animal model studies in which R-type LPS induces protection against heterologous gonococcal challenge in mice and

in embryos obtained from immunized hens (Diena et al., 1978). It was found that hens are excellent producers of antibodies to R-type LPS (Wallace et al., 1978). Antisera produced by immunization of hens with R-type LPS agglutinate N. gonorrhoeae colony-types 1 to 4 and can be used to identify primary isolates and secondary cultures of N. gonorrhoeae (Malysheff et al., 1978; Wallace et al., 1978).

Gonococci can be separated antigenically into at least six serogroups, based on a cell wall polysaccharide which has been shown to be derived from gonococcal LPS; these acidic polysaccharides have been designated Gc antigens (Apicella, 1974; Apicella, 1976; Apicella and Gagliardi, 1979). Apicella and Gagliardi (1979) studied the antigenic structure of the polysaccharide component of gonococcal LPS by immunodiffusion and an enzyme-linked immunosorbent assay. These studies indicated that each gonococcal LPS-derived polysaccharide contains a serogroup-specific determinant which is analogous to the O-specific antigen. In addition, these polysaccharides contain non-serogroup determinants which are heterogeneous. There are at least two sets of non-serogroup determinants; one set is common to all six gonococcal serogroup polysaccharides, whereas the second set is shared by three of the six serogroups. These studies indicated the antigenic complexity of gonococcal LPS.

A noteworthy finding is that the receptor site for the R-type pyocines is in the LPS of Pseudomonas aeruginosa (Govan, 1974; Koval and Meadow, 1977) and a similar location has been suggested for N. gonorrhoeae (Sidberry and Sadoff, 1977).

It was reported that wheat germ agglutinin (WGA) could specifically identify clinical isolates of N. gonorrhoeae (Schaefer, Keller and Doyle, 1979). WGA was also found to be specific for

N-acetyl glucosamine; this compound specifically blocked WGA-mediated agglutination of gonococcal strains. Both gonococcal and meningococcal LPSs contain large amounts of N-acetyl glucosamine (Jennings et al., 1973; Wiseman and Caird, 1977); Frasch (1980) suggested that the specific outer membrane component through which wheat germ lectin interacts to effect agglutination is most likely LPS.

1.3 Outer membrane proteins

Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis of the isolated gonococcal outer membrane demonstrated the relative simplicity of its protein spectrum. Proteins with apparent molecular weights of 34 500, 22 000 and 11 500 constituted the major part of the protein component of the outer membrane; the 34 500 dalton protein accounted for over 60 per cent of the total outer membrane protein and has been termed major outer membrane protein (MOMP) (Johnston and Gotschlich, 1974). It has also been referred to as principal outer membrane protein (POMP) (Buchanan et al., 1977) or protein I (Heckels, 1977).

In order to provide sufficient outer membrane complex containing MOMP for detailed serological analysis, whole cells were extracted with lithium acetate buffer containing EDTA (Johnston, Holmes and Gotschlich, 1976). MOMP in this material could be enriched further by gel chromatography followed by isoelectric precipitation. On the basis of MOMP (the serotype antigen), the outer membrane complex could be used to assign clinical isolates of N. gonorrhoeae into 16 distinct serotypes. These investigators also demonstrated that the apparent molecular weight of MOMP was

strain dependent.

Heckels (1977) used a similar procedure to that of Johnston et al. (1976) to prepare outer membranes; EDTA was omitted from the lithium acetate extraction buffer because preliminary experiments showed that substantial amounts of LPS were also removed from crude cell envelopes by EDTA. The material obtained was similar in gross composition to the complex isolated by Johnston et al. (1976) in that it contained phospholipid, LPS, and relatively few proteins. Treatment with SDS at 37°C revealed five membrane proteins including major bands with molecular weights of 36 500 (protein I) and 24 000 (protein II), whereas treatment at 100°C produced major bands at 36 500 (I) and 29 500 (II*); protein II was therefore replaced by a higher molecular weight protein designated protein II*. Protein I was present in all nine strains examined whereas protein II was present in six of these strains.

The outer membranes could be dissociated by treatment with sodium cholate before separation of the individual surface components (Heckels, 1977). This provided a useful initial separation since protein II and LPS were solubilized by this procedure while protein I was not. Further fractionation was then achieved by gel filtration to give pure LPS and protein II. Thus gonococcal outer membrane can be fractionated by relatively mild methods to give three major surface components: proteins I and II, and LPS.

A protein complex with an apparent molecular weight of 800 000, referred to as outer membrane protein macromolecular complex (OMP-MC), was found in all N. gonorrhoeae strains examined (Newhall et al.,

1980). Further investigation revealed that this protein accounted for approximately 10 per cent of the outer membrane protein mass, was exposed at the cell surface, possessed antigenic activity, could be quantitatively recovered after reduction and alkylation as subunits with a molecular weight of 76 000, and could be purified in the complex form by gel filtration. It was suggested by the authors that the OMP-MC deserves further consideration in studies aimed at describing shared outer membrane antigens, developing vaccines, developing serotype schemes, and inter-relating the structure and function of the gonococcal outer membrane.

Swanson (1978b), in a study of the outer membrane proteins of laboratory strains and clinical isolates of N. gonorrhoeae, found that gonococci from colonies exhibiting optical opacity and dark coloration have surface proteins that are not visualized in isogenic transparent, light-coloured colony-forms. These "colony opacity-associated proteins" have apparent molecular weights varying from 24 000 to 30 000 as determined by SDS-polyacrylamide gel electrophoresis. Their apparent molecular weights are independent of that for their major outer membrane protein. These opacity-associated proteins are more susceptible to hydrolysis by trypsin than is the major outer membrane protein, but gonococci possessing the opacity-associated protein(s) also show enhanced susceptibility of their major outer membrane proteins to the action of trypsin. Lambden and Heckels (1979) found the parent strain of N. gonorrhoeae P9 to be transparent whereas the laboratory strain derived from it formed smooth, intermediate opacity type colonies. Outer membranes from the parent strain contained protein I with a molecular weight

of 36 000 as the only major protein, whereas those from the opacity variants contained one or more of a series of proteins (II*, II*a, II*b, II*c, II*d) in the molecular weight range 29 000-27 500. This emphasizes the need for careful typing of strains to be used in comparative biological experiments. It was suggested by the authors that comparison of surface protein composition is also needed since the number of extra proteins is not quantitatively related to opacity.

The major outer membrane proteins from 10 gonococcal strains were examined after ^{125}I -labelling of the proteins as single bands resolved in SDS-polyacrylamide gel electrophoresis (Swanson, 1979). These ^{125}I -proteins were then treated with either trypsin or alpha-chymotrypsin, and the resultant ^{125}I -peptides were visualized by autoradiography after two-dimensional electrophoretic and chromatographic separation on thin-layer cellulose sheets. Several ^{125}I -peptides were present in all the major outer membrane proteins examined. The presence or absence of additional ^{125}I -peptides segregated the major proteins into two groups. One group consisted of major proteins with molecular weights of 34 000 or 33 000; major proteins with molecular weights of 32 000 constituted the other group. Two beta-lactamase-producing gonococcal isolates were examined: although their major outer membrane proteins were identical in apparent molecular weights and alpha-chymotryptic ^{125}I -peptide fingerprints they contained ^{125}I -peptides not found in other gonococcal major proteins. No ^{125}I -peptide differences were found among the major outer membrane proteins of strain F62 gonococci that exhibited differences in piliation and/or colony opacity

characteristics. Several colony opacity-associated proteins, which are among a group of "minor" proteins that exhibit heat modification of their apparent molecular sizes, were subjected to ^{125}I -peptide mapping of protein bands separated by SDS-polyacrylamide gel electrophoresis (Swanson, 1980). A relatively high apparent degree of structural homology was found among these heat-modifiable proteins from different strains of opaque gonococcal colonies. There was also some apparent structural homology for ^{125}I -peptides of heat-modifiable versus major proteins of the gonococcal outer membrane.

Heckels (1978) investigated the topographical distribution of the outer membrane protein antigens. The autoradiography of an SDS-polyacrylamide gel of gonococci labelled with ^{125}I -lactoperoxidase revealed that protein I was always present and heavily labelled. The second major protein (II) was present in variable amounts and labelling was proportional to the amount present. The absence of protein III from the cell surface was evidenced by its poor labelling. Protein I is therefore a major component of the cell-surface. The absence of protein III and the variability of protein II on the gonococcal surface suggest that perhaps protein I is the most promising candidate of the outer membrane proteins to be of value in the current search for a gonococcal vaccine, as pointed out by the author.

Much work has been done on the qualitative and quantitative analyses of gonococcal outer membrane proteins, but as yet very few investigations of the architecture of these proteins in the

membrane matrix have been reported. Cross-linking studies of outer membrane proteins revealed the ability of protein I to become cross-linked to peptidoglycan (Heckels, 1979); this and the finding that protein I is a major component of the gonococcal cell-surface (Heckels, 1978) demonstrate a transmembrane arrangement for this protein. This strongly suggests that protein I may play a role analogous to the transmembrane proteins of E. coli which form hydrophilic diffusion pores (DiRienzo, Nakamura and Inouye, 1978). In a separate study, cross-linking of isolated outer membranes yielded dimers and trimers of the major outer membrane protein (Leith and Morse, 1980). The observation that protein I may be cross-linked to protein II in N. gonorrhoeae indicates that, at least in certain regions of the outer membrane, proteins I and II are found in close association. Heckels (1979) reported the failure to cross link any of the proteins (II*, II*a, II*b, II*c, II*d) directly to peptidoglycan. Their failure to link to peptidoglycan even through protein I suggested to the author that the two types of protein occur in discrete regions of the outer membrane. McDade and Johnston (1980) reported results of cross-linking experiments strongly suggesting that two of the major proteins of the gonococcal outer membrane (proteins 1 and 3) form a hydrophobically associated trimeric unit in situ which can be stabilized by selective cross-linking reagents. Results substantiated that these proteins are responsible for imparting serotypic specificity. These analyses of the architecture of the outer membrane proteins do not concur exactly; further studies should in time confirm some of these findings.

The purification of a gonococcal antigen designated "L-antigen" was reported by Chen et al. (1977). The purified antigen was heat labile, trypsin-sensitive, and had an approximate molecular weight of 38 500, as determined by SDS-polyacrylamide gel electrophoresis. The L-antigen is apparently distinct from the pili and major outer membrane protein antigens. The latter were strain-specific (Johnston et al., 1976), while the L-antigen was species-specific (Gaafar and D'Arcangelis, 1976). Most isolates failed to express the L-antigen when grown in vitro (Chen et al., 1977).

Gonococci grown in guinea pig chambers were reported to possess one or more envelope-associated protein components that were not detectable in the parent strain maintained in vitro (Penn et al., 1976). The relationship of this protein(s) to the L-antigen or other envelope components is not known. As opposed to strain P9 maintained in the laboratory by repeated subculture, Heckels and Everson (1978) observed that the parent strain P9 maintained as a freeze-dried sample with minimum subculture possessed a major outer membrane protein with a molecular weight of 60 000. The reappearance of such components may account for reports of new surface antigens on gonococci after growth of laboratory strains in vivo in guinea pig chambers (Penn et al., 1976). However, only one strain was examined by Heckels and Everson (1978), and it is not known whether the loss of outer membrane proteins with repeated subculture is a generalized phenomenon or the result of selecting a spontaneous mutant. Nevertheless, variants lacking particular outer membrane proteins should be valuable in comparative experiments to correlate biological properties with particular surface components.

1.4 Peptidoglycan

The chemical composition of purified gonococcal peptidoglycan was initially reported by Hebelers and Young (1975), and subsequently confirmed by Wolf-Watz et al. (1975). Both groups of investigators found muramic acid, glucosamine, alanine, glutamic acid, and diaminopimelic acid in approximate molar ratios of 1:1:2:1:1, respectively. Identical composition and molar ratios were found in the peptidoglycan isolated from colony-types 1 to 4 organisms. Analysis of acid hydrolysates of purified gonococcal peptidoglycan revealed that 96 per cent of the total weight of the sample can be accounted for as wall amino acids and amino sugars (Hebelers and Young, 1976a). Gonococcal peptidoglycan was completely solubilized by lysozyme (100 µg/ml), but not by lysostaphin.

Trace amounts of aspartic acid, glycine, and threonine were observed in purified gonococcal peptidoglycan (Hebelers and Young, 1976a). Wolf-Watz et al. (1975) also reported the presence of trace amounts of aspartic acid and glycine. These trace amino acids were not removed by treatment with either trypsin or pronase. Whether these amino acids represent accessory cell wall polymers or contamination is not known. No covalently attached lipoprotein has been reported (Wolf-Watz et al., 1975; Hebelers and Young, 1976a). The absence of molecules, analogous to the lipoprotein found in E. coli, which link the peptidoglycan with the outer membrane is thought to be responsible for the apparent loose association of the outer membrane to the peptidoglycan as seen in electron micrographs of N. gonorrhoeae (Wolf-Watz et al., 1975).

The proportion of peptidoglycan present in N. gonorrhoeae is

similar to that in other Gram-negative bacteria. Hebeler and Young (1976a) determined that the peptidoglycan represents 1 to 2 per cent of the dry weight of the cells; no significant differences are observed between the various colony-types. However, growth pH was reported to influence markedly the composition of the cell envelope of N. gonorrhoeae (Hebeler et al., 1978). This report was subsequently confirmed by Hebeler et al. (1979). The peptidoglycan in cells grown at pH 7.2 accounted for 1 to 2 per cent of the dry weight of the cells. However, in cells cultured at pH 6.0, the dry weight of peptidoglycan increased to 4-13 per cent. Preliminary investigations indicated that the apparent increase in weight was strain dependent and was due in part to associated protein(s). The data suggest that the protein is covalently attached to the peptidoglycan; the peptidoglycan-protein complex could not be dissociated by additional extraction with SDS, lithium chloride, or EDTA.

The chain length of the glycan backbone of peptidoglycan has been determined by reducing group analysis (Hebeler and Young, 1976a). It was calculated that the average chain length of the glycan backbone is between 80 and 110 disaccharide units.

The degree of cross-linking in gonococcal peptidoglycan has been the subject of very few investigations. Guymon, Walstad and Sparling (1978) determined the relative degrees of cross-linking among an isogenic set of N. gonorrhoeae strains, and made the interesting observation that low-level non-specific resistance to several antibiotics correlated with an increase in the extent of peptidoglycan cross-linking. However, their data could not be

interpreted in terms of the absolute extent of cross-linking. More recently, Rosenthal, Wright and Sinha (1980) examined the extent of peptide cross-linking in peptidoglycan isolated from various strains of N. gonorrhoeae. Although there were subtle, strain- and medium-dependent differences in percentage cross-linking, these values varied only over a narrow range (36 to 44 per cent); this is a relatively high degree of peptide cross-linking for a Gram-negative bacterium. The authors also confirmed previous observations (Guymon et al., 1978) that the extent of peptidoglycan cross-linking among isogenic gonococci was higher in strains carrying loci that govern increased resistance to multiple drugs.

The high autolytic activity displayed by N. gonorrhoeae during all stages of growth (Hebeler and Young, 1975) prompted Hebeler and Young (1976a) to postulate that peptidoglycan turnover may occur. Using gonococci labelled during growth in medium containing ³H-diaminopimelic acid, they showed that peptidoglycan turnover follows first order kinetics with approximately a 50 per cent turnover rate per generation. ³H-diaminopimelic acid specifically labels the tetrapeptide chain of the peptidoglycan, and therefore these data reflect the turnover of the tetrapeptide portion of the peptidoglycan.

Gonococci appear to be unusual among Gram-negative bacteria in that they exhibit extensive turnover of their peptidoglycan layer during exponential growth (Guymon et al., 1978; Rosenthal, 1979), and shed the soluble peptidoglycan fragments into the culture medium.

It was determined that the major autolysin of N. gonorrhoeae is an N-acetylmuramyl L-alanine amidase; it was solubilized from gonococcal envelopes by extraction with Triton X-100 and NaCl (Hebeler and Young, 1976b). Significant amidase activity in gonococci was also demonstrated by Wegener, Hebeler and Morse (1977). It has been suggested that gonococci may also possess glycan-splitting (hexaminidase) activity (Hebeler and Young, 1976b; Wegener et al., 1977). Preliminary analysis of the soluble peptidoglycan detected in gonococcal supernatants suggested that turnover and release of peptidoglycan by growing gonococci was mediated by both glycan-splitting (hexaminidase) and peptide-splitting (N-acetylmuramyl L-alanine amidase) activities (Rosenthal, 1979).

1.5 Capsule

The presence or absence of a capsule on N. gonorrhoeae has been debated for many years. The finding that meningococci have a polysaccharide capsule which provides a basis for serogrouping suggests the possibility that gonococci may be capsulate. The chemical structure of many of the meningococcal capsular polysaccharides has been determined and found to consist of one or two sugars (Liu et al., 1971a; Liu et al., 1971b; Bhattacharjee, Jennings and Kenny, 1974; Bundle, Jennings and Kenny, 1974; Bhattacharjee et al., 1976).

Freshly isolated strains of N. gonorrhoeae were reported to possess a capsule demonstrable by light microscopy with India ink as a negative stain, and by electron microscopy of cells exposed to hyperimmune serum (Hendley et al., 1977). These observations

were confirmed by three other groups of investigators (James and Swanson, 1977; Richardson and Sadoff, 1977; DeHormaeche, Thornley and Glauert, 1978). Capsular synthesis is apparently dependent upon the presence of undefined medium components and the growth phase of the organism. The addition of casein hydrolysate to standard media stimulates capsule production by organisms in the logarithmic phase of growth (Hendley et al., 1977). Richardson and Sadoff (1977) reported that capsule production was most apparent when gonococci were cultivated on agar medium in the presence of a strain of microaerophilic "viridans streptococci". It is not known whether the streptococci produce a factor that stimulates capsule synthesis, removes chemicals, or otherwise alters the composition of the medium to favour capsule synthesis.

According to James and Swanson (1977), the capsule is loosely associated with the cell-surface and can be easily removed by mild shearing. Although the capsule is most evident on recent clinical isolates, it is also present on the surface of laboratory strains (James and Swanson, 1977). Cells of the virulent and avirulent colony-types produce capsules (James and Swanson, 1977; Richardson and Sadoff, 1977).

A method using India ink in electron microscopic preparations was used to study bacterial capsules (Melly et al., 1979). Capsules were demonstrated on Streptococcus pneumoniae and Staphylococcus aureus. No true capsules were definitively identified on N. gonorrhoeae grown in vitro or obtained from human urethral exudates. The method described does not require the use of specific anticapsular antibody or specific polysaccharide stains; it was

suggested by the authors that it permits the detection of artefacts that may, by the use of light microscopic methods, be misinterpreted as capsules.

Wheat germ agglutinin, having specificity for N-acetyl glucosamine, agglutinates non-capsulate Neisseria strains through interaction with lipopolysaccharide in the outer membrane (Frasch, 1980). This lectin agglutinated known non-capsulate strains of N. meningitidis, but failed to agglutinate capsulate strains of all serogroups tested. In contrast, N. gonorrhoeae strains were strongly agglutinated providing additional evidence for the absence of a capsule on N. gonorrhoeae (Schaefer, Keller and Doyle, 1979).

If the capsule does exist, its true chemical nature and homo- or heterogeneity of capsular antigens remain to be discovered.

1.6 Autolysis

N. gonorrhoeae does not survive for long periods after the cessation of growth. The decrease in viability is often correlated with cellular lysis which can occur following the depletion of glucose in the medium (Morse and Bartenstein, 1974). The autolysis of N. gonorrhoeae has been studied by measuring the optical density of washed cells suspended in an appropriate buffer as a function of time (Hebeler and Young, 1975; Elmros, Burman and Bloom, 1976a; Wegener et al., 1977).

Various environmental conditions influence the rate of autolysis of gonococci (Elmros et al., 1976a). Whole cell autolysis was enhanced by K^+ (Hebeler and Young, 1975) and markedly inhibited by divalent cations (Ba^{++} , Ca^{++} , Cu^{++} , Hg^{++} , Mg^{++} , Mn^{++}) (Elmros et al., 1976a; Wegener et al., 1977). The inhibitory

effect of divalent cations on autolysis can be reversed by the addition of a chelating agent such as EDTA. Trivalent cations (Fe^{+++} and Al^{+++}) showed only slight inhibition of autolysis (Wegener et al., 1977). The nature of the buffer is another important variable. Buffers such as Tris, which chelate divalent cations, yield faster rates of autolysis than buffers such as HEPES, which do not chelate divalent cations as readily (Wegener et al., 1977; Morse, 1979). Whole cell autolysis is optimum at pH 9.0 and 40°C (Hebeler and Young, 1975). The observation that the rate of autolysis is temperature dependent and can be irreversibly inactivated by heating, at 80°C for 10 minutes, suggests that autolysis is enzyme-mediated (Hebeler and Young, 1975; Wegener et al., 1977).

Incubation of gonococci with chloramphenicol or rifampicin suppresses autolysis in buffer (Morse and Bartenstein, 1974), but does not eliminate peptidoglycan hydrolysis (Wegener et al., 1977); this indicates that peptidoglycan hydrolysis per se does not account for autolysis, and that other factors regulate a second event which is required for autolysis.

The biochemical mechanisms of the autolytic process have not been resolved. The demonstration of phospholipase A activity in the envelope of N. gonorrhoeae has been reported (Senff et al., 1976). The phospholipase A was heat-sensitive, required Ca^{++} for activity, and exhibited optimal activity at pH 8.0 to 9.0; this is also the optimal pH for autolysis. Lysophospholipase has also been reported in the outer membrane of N. gonorrhoeae (Wolf-Watz et al., 1975). The relationship between conditions

which induce or inhibit cell lysis, and those which promote phospholipid hydrolysis in N. gonorrhoeae was investigated (Cacciapuoti, Wegener and Morse, 1978). Suspension of exponential-phase gonococci in buffer in the absence of divalent cations resulted in autolysis but not in phospholipid hydrolysis. The addition of Ca^{++} or Mg^{++} to the buffer inhibited autolysis and markedly stimulated phospholipid hydrolysis. These data indicated that phospholipid hydrolysis per se does not account for autolysis, although it may cause loss of integrity of the cell envelope.

Activation of autolysis may produce cells with damaged envelopes which may remain viable due to stabilization with divalent cations (Elmros, Sandstrom and Burman, 1976b). Autolytic activity may enhance the conversion of gonococci to L-phase variants (Elmros et al., 1976c).

1.7 L-forms of N. gonorrhoeae

Wall-defective variants are microorganisms that have undergone changes in morphology, physiology, and/or cultural characteristics as a result of damage to or deficiency of their cell walls (McGee et al., 1971). The use of the term "variant" does not imply a genetic change. Various types of wall-defective variants of N. gonorrhoeae have been reported, and because they have often been misinterpreted as L-forms, McGee et al. (1971) defined criteria for wall-defective variants. Accordingly, L-phase variants are wall-defective variants that can replicate serially as non-rigid cells. On solid media, they produce distinctive colonies composed of a core growing into the agar and a superficial growth around the core giving them a "fried egg" appearance. Individual organisms

vary in size from "large bodies", larger than the parent bacterial form, to "small bodies", smaller than some viruses. They are indifferent to penicillin, and are usually spherical or pleomorphic. Osmotic stabilization for optimum growth in vitro may be required. The "L-form" refers solely to the distinct morphology of colonies composed of L-phase variants.

Due to the highly autolytic nature of the gonococcus, it is not surprising that wall-defective variants have been observed. Dienes, Bandur and Madoff (1964) described the spontaneous induction of wall-defective variants in gonococcal colonies undergoing autolysis. The tendency to develop wall-defective variants varied within individual colonies of a single strain as well as between strains themselves (Dienes et al., 1964).

Wall-defective variants were also produced following exposure to penicillin or high concentrations of exogenous glycine (Dienes et al., 1964), but all attempts at subculture failed. Using a penicillin-gradient technique, Roberts (1966) successfully induced L-forms in low frequencies in a medium osmotically stabilized with 10 to 20 per cent sucrose. After ten serial passages in the presence of penicillin, reversion to the parental form when transferred to penicillin-free medium became infrequent. Sucrose and serum were found to be necessary medium constituents for the in vitro production of gonococcal L-forms. Lawson and Douglas (1973) and Bacigalupi and Lawson (1973) elucidated the cultural conditions necessary for massive conversion of N. gonorrhoeae to L-forms. They found that the substitution of dialyzed polyvinylpyrrolidone for sucrose in L-medium containing penicillin allowed

higher frequencies of L-form induction, which approached 100 per cent in some strains. These findings were confirmed by Geary and Waitkins (1977).

The biological significance of L-forms of N. gonorrhoeae remains to be elucidated.

2. Deoxyribonucleic acid

2.1 Chromosomal

The chromosome of the gonococcus has approximately 1.5×10^6 nucleotide pairs and a molecular weight of 9.8×10^8 (Kingsbury, 1969; Morse, 1979). A comparison of genome sizes suggests that the gonococcus is limited in its genetic potential. The relative size of the gonococcal genome may be typical for a pathogenic organism which has become highly adapted to growth in its host. The chemical composition of gonococcal DNA has been determined and found to be similar to that reported for other neisseriae (Lee, Wahl and Barbu, 1956; Catlin and Cunningham, 1961).

2.2 Plasmid

Plasmids are extrachromosomal genetic elements of bacteria that are capable of autonomous replication. They are in addition to the normal genetic pool and are not essential, under most circumstances, for the growth of the host. Many strains of N. gonorrhoeae possess phenotypic properties, such as multiple drug resistance and the presence of pili, which have been shown to be plasmid mediated in other bacterial species (Elwell and Falkow, 1977). Two plasmids have been described in N. gonorrhoeae, a small 2.6×10^6 dalton (2.6 Mdal) multicopy plasmid (Mayer, Holmes

and Falkow, 1974) and a large 24.5×10^6 dalton (24.5 Mdal) plasmid (Stiffler et al., 1975). No phenotype could be correlated with the presence of either plasmid (Mayer et al., 1974; Stiffler et al., 1975; Biswas, Comer and Sparling, 1976).

Beta-lactamase-producing gonococci were simultaneously isolated in England and the United States (Phillips, 1976; Ashford, Golash and Hemming, 1976). In addition to the phenotypically cryptic 2.6 Mdal plasmid present in all strains, beta-lactamase-producing strains originating in the Far East carried a 4.4 Mdal R plasmid while strains isolated in England carried a 3.2 Mdal R plasmid (Roberts and Falkow, 1977). Moreover, 50 per cent of the strains carrying the 4.4 Mdal R plasmid also were found to harbour the 24.5 Mdal plasmid (Roberts, Elwell and Falkow, 1977); none of the strains carrying the 3.2 Mdal R plasmid carried the large plasmid (Roberts and Falkow, 1977). The large 24.5 Mdal plasmid has been shown to have sex factor activity and can mediate transfer of itself, R plasmids, and chromosomal genes (Baron et al., 1977; Eisenstein et al., 1977; Kirven and Thornsberry, 1977; Roberts et al., 1977; Roberts and Falkow, 1977, 1978). Roberts, Piot and Falkow (1979) have examined the molecular relationships between the various large gonococcal plasmids from strains of N. gonorrhoeae from various geographic areas isolated between 1940 and 1978. The 24.5 Mdal plasmid appears to be common only in certain geographic areas, and the results of hybridization and restriction enzyme studies suggest that these large plasmids represent a group of closely related molecules.

2.3 Mechanisms of genetic exchange

There are two mechanisms of genetic exchange in N. gonorrhoeae, transformation and conjugation. Genetic transformation is the process in which one bacterium takes up, integrates, and expresses (and is thereby transformed by) naked DNA from another organism (Sparling, Biswas and Sox, 1977). The phenomenon was originally discovered by Griffith (1928). Transformation of Neisseria species was first reported by Alexander and Redman (1953), and later by Catlin (1960) and Catlin and Cunningham (1961). N. gonorrhoeae was not used as recipient until 1966 when Sparling demonstrated that gonococci are also transformable.

In most bacteria which are capable of undergoing genetic transformation, the competent state "is a transitory state of the recipient population, and its duration is restricted to a small fraction of the growth cycle" (Hayes, 1970). In this respect, N. gonorrhoeae and other neisseriae are atypical. All strains of N. gonorrhoeae examined have been found competent for genetic transformation (Sparling, 1966; Sparling et al., 1977). However, transformation frequencies are significantly higher ($\geq 10^3$ -fold) with piliate cells than with non-piliate cells (Sparling, 1966); non-piliate gonococci are usually relatively incompetent. Non-piliate cells treated with EDTA or lysozyme to increase cell envelope permeability showed a moderate increase in phenotypic competence, however this treatment also resulted in a marked decrease in viability (Biswas et al., 1977). Piliate gonococci can be transformed throughout all phases of growth, and competence remains virtually unchanged throughout the growth cycle (Sparling,

1966; Biswas et al., 1977). It was found that cells were somewhat more competent when grown and transformed in complex medium than when grown and transformed in defined medium (Biswas et al., 1977).

Competence appears to be a stable property of piliate gonococci. Siddiqui and Goldberg (1975a) reported that washing competent piliate cells with phosphate-buffered saline resulted in a 1000-fold decrease in the transformation frequency. Re-suspension of these washed cells in culture supernate restored normal transformation frequencies. However, the presence of chloramphenicol inhibited the ability of the culture supernate to enhance competence. Siddiqui and Goldberg (1975a) postulated the existence of a soluble competence-enhancing factor, and suggested that competence in N. gonorrhoeae required protein synthesis for its expression. Unfortunately, other investigators were not able to confirm these data (Biswas et al., 1977; Sparling et al., 1977).

Biswas et al. (1977) defined the conditions which would enhance transformation of N. gonorrhoeae. The addition of either Mg^{++} or Ca^{++} markedly stimulated the efficiency of transformation, monovalent cations such as NH_4^+ , Na^+ , and K^+ also stimulated transformation. Divalent cations such as Co^{++} , Cu^{++} , and Mn^{++} failed to stimulate transformation. These data are similar to those reported by Barnhart and Herriott (1963) with Haemophilus influenzae. These workers postulated that the cations neutralized the net negative surface charge of the cell, allowing the negatively-charged DNA to make contact.

Transformation occurs over a temperature range of 8 to 42°C, but is optimum between 36 and 37°C (Sparling, 1966). The efficiency of transformation is also dependent upon the pH; Biswas et al. (1977) reported an optimum pH range of 7.0 to 8.0.

The degree of competence of gonococci does not correlate with their ability to bind DNA (Biswas et al., 1977). The physical binding of DNA can be dissociated from its uptake by treatment of the cells with deoxyribonuclease (DNAase). Biswas et al. (1977) found that only 0.5 per cent of added DNA was taken up into a DNAase-resistant state, and this was only observed in competent colony-type 1 cells. No DNAase-resistant uptake was observed with colony-type 4 cells, and transformation did not occur. DNAase-resistant uptake and transformation efficiency followed essentially parallel curves, with saturation at about 0.5 µg of DNA per ml. More recently, it was observed that although competent gonococci were capable of adsorbing both homologous and heterologous DNA, DNAase-resistant uptake was a small fraction of the homologous DNA only (Dougherty, Asmus and Tomasz, 1979).

Uptake of DNA is dependent upon its physical state, with markedly reduced uptake if DNA is single rather than double stranded (Hotchkiss and Gabor, 1970). Sparling et al. (1977) obtained similar data for the gonococcus; single stranded DNA is about 1 per cent as active in transformation as double stranded DNA. Very little is known about the fate of DNA during and after uptake by N. gonorrhoeae. In other systems, donor DNA activity is lost shortly after uptake owing to its conversion into the single stranded state prior to being integrated into the bacterial

genome (Hotchkiss and Gabor, 1970). However, this phenomenon (eclipse phase) is apparently lacking in N. gonorrhoeae since the activity of donor DNA remains unchanged when extracted from DNAase-treated, washed cells, at short intervals (Sparling et al., 1977). In a recent report, Biswas and Sparling (1981) concluded that the bulk of donor DNA in N. gonorrhoeae enters the cell in double stranded form, and that most donor DNA is not converted to single strands after uptake.

Sparling et al. (1977) investigated whether pili serve as receptors for DNA during transformation. They were unable to demonstrate binding between ^3H -DNA and purified unlabelled pili, or between unlabelled DNA and ^{125}I -pili. The role of pili was ruled out by the finding that bound DNA could not be eluted when cells from both colony-types 2 and 4 were subjected to strong shearing forces (Dougherty et al., 1979). Thus, the direct involvement of pili remains speculative, and the explanation for the ability of all piliate gonococcal strains to take up DNA remains unknown. In a study of the effect of heterologous and homologous DNA on the genetic transformation of gonococci, Dougherty et al. (1979) suggested the presence of two types of DNA binding sites or receptors on the surface of competent gonococci. Homologous and heterologous DNA adsorb to receptor 1 and are accessible to external DNAase; receptor 2 is specific for homologous DNA or DNA from taxonomically related species, a portion of which becomes resistant to DNAase treatment. However, the presence of two different types of DNA receptors on the surface of competent gonococci remains speculative until these binding sites

are characterized.

Transformation may occur in vivo. Sarubbi and Sparling (1974) demonstrated that genetic exchange took place when two strains of N. gonorrhoeae carrying different chromosomal antibiotic resistance markers were co-cultivated in liquid medium. However, the direction of gene transfer was dependent on the colony-types of the two strains. It was determined by auxotyping that many cases of gonorrhoea (approximately 25 per cent) represent infection with more than one auxotype (Short et al., 1977). Thus, although direct proof is lacking, the opportunity and conditions are present for in vivo transformation to occur.

Intragenetic transformation between N. gonorrhoeae and other species of Neisseria can occur. Sparling et al. (1977) observed that transformation efficiencies were affected both by species and the particular marker studied. The most efficient heterologous cross was between N. gonorrhoeae and N. meningitidis, and approached the efficiency of the homologous cross with respect to most markers studied (Siddiqui and Goldberg, 1975b; Wood and Brownell, 1975; Sparling et al., 1977).

Genetic transformation has been used to map a number of chromosomal antibiotic resistance genes in N. gonorrhoeae (Sparling, 1977). Resistance to a variety of antibacterial agents was found to be controlled by six genetic loci which fell into three linkage groups (Maier, Zubrzycki and Coyle, 1975a; Maier et al., 1975b). Group I contains loci determining resistance to streptomycin, tetracycline, chloramphenicol, and erythromycin. This region was also shown to include loci that specify resistance

to rifampin and spectinomycin (Sarubbi, Blackman and Sparling, 1974). Group II contains the multiple resistance locus (mtr) which confers a low level of resistance to a variety of agents. Group III contains a locus (Pen) that specifies resistance to relatively high levels of penicillin. Genetic transformation has also been used, but less extensively, for the mapping of nutritional markers (Steinberg et al., 1979).

Sox, Mohammed and Sparling (1979) obtained data suggesting the transfer of penicillin resistance plasmids between gonococci by transformation. The transformants obtained in this study contained penicillin resistance plasmids which were either larger or smaller than the donor plasmid, possibly because of exposure to surface endonucleases. It has been shown by DNA-DNA hybridization that the 4.4 and 3.2 Mdal gonococcal R plasmids are closely related (Roberts, Elwell and Falkow, 1977). These data put together suggested to Sox et al. (1979) that penicillin resistance plasmids may have been transferred between gonococci by transformation, with formation of a 3.2 Mdal R plasmid by a specific deletion from a 4.4 Mdal R plasmid.

A conjugation system has been reported in N. gonorrhoeae. Strains which carry a 24.5 Mdal plasmid have been shown to mobilize the 4.4 Mdal penicillin resistance plasmid to penicillin-sensitive recipients (Eisenstein et al., 1977; Roberts and Falkow, 1977; Sox et al., 1978; Biswas, Blackman and Sparling, 1980). Roberts and Falkow (1979) were able to detect R plasmid transfer in guinea pig subcutaneous chambers, only when the donor strain carried the 24.5 Mdal plasmid along with the R factor; transfer could be detected in the absence of antibiotic pressure.

In conjugation, genetic transfer frequencies into piliate or non-piliate gonococcal strains are identical. There is also no correlation between colony-type and the ability to serve as donor or recipient (Eisenstein et al., 1977; Roberts and Falkow, 1977). Biswas et al. (1980) observed marked differences in penicillin resistance plasmid transfer efficiencies, in isogenic strains varying only in the 28 000 dalton outer membrane heat-modifiable opacity-associated protein. Maximal transfer of the R plasmid was observed between transparent donors and recipients, lacking appreciable amounts of the 28 000 dalton protein. One might have expected this protein to increase the efficiency of conjugation, since it promotes increased adhesion between gonococcal cells (Swanson, 1978a, 1978b) and therefore would be expected to increase formation and stability of mating aggregates.

Roberts and Falkow (1978) have reported the mobilization of the chromosome by the 24.5 Mdal plasmid and transfer of chromosomal genes. Other workers, using essentially the same system, have failed to reproduce their results (Sox et al., 1978). More recently, various studies have confirmed that chromosomal DNA can be exchanged between gonococci by transformation, but not by conjugation (Norlander, Davies and Normark, 1979; Biswas et al., 1980; Steinberg and Goldberg, 1980).

At present, since there still is no known gonococcal bacteriophage, and therefore no system for transduction, transformation remains the only reproducible system for study of the genetics of the gonococcal chromosome.

3. Antimicrobial susceptibility

Shortly after the introduction of sulphonamides, gonococci were found to be sensitive and therapy was revolutionized. Unfortunately, resistance to the sulphonamides developed relatively rapidly, and by the end of World War II was so extensive that sulphonamide therapy was no longer reasonable (Sparling, 1977). Penicillin was introduced at about the same time as sulphonamides were losing their effectiveness. Gonococci were found to be particularly sensitive to penicillin. However, strains with reduced sensitivity were soon reported (Curtis and Wilkinson, 1958; Reyn, Korner and Bentzon 1958) and have continued to increase in prevalence in many areas of the world (Willcox, 1970; Sparling, 1972). These levels of resistance were over 100 times greater than prevailed 20 years earlier, but were still low-level in comparison with the resistance seen in staphylococci and enteric bacteria (Sparling, 1977).

Although the therapeutic outlook has changed for reasons which will be discussed below, penicillin remains the first drug of choice in the treatment of gonococcal infections (Johnston, Kolator and Seth, 1981). In view of this consideration, penicillins and the problems associated with their use will be the main topic dealt with in this section.

Penicillin resistance in N. gonorrhoeae is of two types, chromosomally mediated low-level resistance (Biswas, Comer and Sparling, 1976) and the less common plasmid-mediated high-level resistance which appeared suddenly in 1976 (Ashford et al., 1976; Phillips, 1976). Until recently, the upper limit of chromosomally

mediated resistance has rarely exceeded a minimum inhibitory concentration (MIC) of 2 µg/ml, and even these strains respond to sufficiently large doses of penicillin if given with probenecid (Wilkinson, 1977). In 1961, a survey of strains isolated before treatment in England showed the majority to be fully sensitive to penicillin, only 13.2 per cent having significantly diminished sensitivities with MICs of 0.125 to 1.0 µg/ml. However, the emergence of N. gonorrhoeae strains unusually resistant to penicillin (MIC of 30 µg/ml), although non-beta-lactamase producing, has been reported recently (Shtibel, 1980).

Low-level resistance has been traced to three genetic markers, pen A, mtr, pen B (Maier et al., 1975a; Sparling, Sarubbi and Blackman, 1975). pen A is a specific marker for penicillin resistance, whereas mtr and pen B are non-specific since they confer resistance to several other antibiotics as well. mtr also retards the uptake of crystal violet into cells (Guymon and Sparling, 1975), which implies that this marker reduces the permeability of the cell envelope. In Gram-negative enteric bacteria, one of the functions of the outer membrane may be a non-selective permeability barrier to large molecules (Leive, 1974). High molecular weight antibiotics such as actinomycin, erythromycin and rifampin have poor activity against many Gram-negative bacteria owing to poor penetration of the outer membrane, a property often referred to as intrinsic resistance (Leive, 1974; Scudamore, Beveridge and Goldner, 1979a). Traditionally, N. gonorrhoeae is atypical in this respect, showing susceptibility to most antimicrobial agents; this suggests that the outer membrane is relatively penetrable (Scudamore, Beveridge and Goldner, 1979b).

The non-specificity of the mtr and pen B markers suggests that they might function by altering the outer membrane to enhance its properties as a passive penetration barrier in N. gonorrhoeae. However, Scudamore et al. (1979b) found no experimental evidence to support this hypothesis.

A type of penicillin resistance described in E. coli is due to a mutation which yields a penicillin-binding protein (FBP) with diminished affinity for the antibiotic (Spratt, 1978). Similarly, the inner (cytoplasmic) membrane of N. gonorrhoeae has been implicated in penicillin resistance through studies of the comparative penicillin binding in susceptible and resistant strains. The isolated inner membranes of the resistant strains were reported to have a lower binding capacity, and in one resistant mutant there was evidence of altered FBPs (Rodriguez and Saz, 1978). However, it remains uncertain whether these changes were related to specific (pen A) or to non-specific (mtr, pen B) antibiotic resistance. On the other hand, it is interesting to note that penicillin susceptibility associated with most strains isolated from patients with disseminated gonococcal infection (DGI) cannot be correlated with any specific changes in their FBP patterns (Nolan and Hildebrandt, 1979).

The plasmids involved in the less common high-level penicillin resistance have already been discussed (p.33). The epidemiological data suggest that there are two separate endemic zones of infection, one in the Far East, the other in West Africa (Johnston et al., 1981). Penicillinase-producing N. gonorrhoeae (P.P.N.G.) isolated in, or epidemiologically linked with the Far East are relatively

resistant to tetracycline in vitro, are phenotypically wild-type or proline-dependent auxotypes; these strains carry a 4.4 Mdal plasmid coding for beta-lactamase production. In contrast, P.P.N.G. epidemiologically linked with West Africa are more susceptible to tetracycline, require arginine for growth, and carry a 3.2 Mdal plasmid. Moreover, 43 per cent of the Far Eastern strains, but none of those from West Africa, have an additional 24.5 Mdal conjugative plasmid.

Epidemics caused by P.P.N.G. have been reported in a number of countries including the United Kingdom (Percival et al., 1976), and in the Netherlands P.P.N.G. are reported to have become endemic (Bijkerk, 1980; Nayyar et al., 1980; van Embden et al., 1980). There has been a steady increase in reported cases of P.P.N.G. in the United Kingdom between 1977 and 1980 (Johnston et al., 1981), illustrating the potential hazard these resistant strains represent.

It is interesting to note that the beta-lactamase of N. gonorrhoeae is similar to the TEM beta-lactamase of enteric species in that it is active against penicillin, ampicillin and cephaloridine (Phillips, 1976). More recently, the beta-lactamases of 18 P.P.N.G. strains were found to be of the TEM-1 type (van Embden et al., 1980). The gene specifying the TEM beta-lactamase resides upon a common sequence of DNA (TnA) within the 3.2 Mdal plasmid; it has the ability to be excised from one plasmid and become inserted into a recipient plasmid (Heffron, Rubens and Falkow, 1975). This genetic "promiscuity" has been offered as an explanation of the widespread presence of the TEM beta-lactamase gene among R plasmids of the Enterobacteriaceae and other bacterial

species. It has also been demonstrated that the gonococcal resistance plasmids carry about 40 per cent of the transposable DNA sequence, TnA (Roberts, Elwell and Falkow, 1977; van Embden et al., 1980).

Roberts et al. (1977) have clearly shown that the gonococcal R plasmids were acquired from a foreign source. The fact that these plasmids are closely related to the Haemophilus plasmid does not necessarily suggest that a Haemophilus species was the direct source. Roberts et al. (1977) suggest that the gonococcal plasmids merely represent an extension of the enteric R plasmid pool. More recently, the 3.2 Mdal plasmid was found in two isolates of H. parainfluenzae (van Embden et al., 1980).

Branhamella catarrhalis is a common component of the human indigenous flora of the nasopharynx; the occasional beta-lactamase-producing strains of B. catarrhalis might be a potential source of beta-lactamase genes transferable to genera like Neisseria and Haemophilus (Malmvall, Brorsson and Johnsson, 1977). However, van Embden et al. (1980) found no indication that penicillinase production, in two strains of B. catarrhalis they examined, is transferable or plasmid mediated. Furthermore, the beta-lactamase in these strains was not a TEM-like enzyme. Similar results have been obtained with the commensal Neisseria perflava (Piot, Roberts and Ninane, 1979).

CLINICAL ASPECTS AND HOST-PARASITE INTERACTIONS

1. Clinical presentation1.1 Uncomplicated gonococcal infection

In men, acute anterior urethritis is the most common manifestation of gonococcal infection (Handsfield, 1978). The patient complains of urethral discharge and an often mild dysuria in about 90 per cent of cases. If infection has spread proximally to the posterior urethra there may be symptoms of frequency of micturition, urgency, and painful erections. Clinical examination may reveal a reddened urethral meatus with a purulent or muco-purulent discharge. Inguinal lymph nodes may be enlarged on both sides. Examination of the urine by the two glass test will show pus in the first glass if the anterior urethra is mainly affected, or in both glasses if the posterior urethra and/or bladder is involved. If the inflammatory process is less severe, evidence of urethritis may take the form of finding "threads" (i.e. casts of urethral glands composed of pus cells and desquamated tubular cells) in the urine (Robertson et al., 1980).

It is now clear that a considerable number (possibly as many as 15 per cent in some localities) of males with urethral gonorrhoea have few symptoms if any (Nielsen, S ndergaard and Ullman, 1975).

Women with gonorrhoea are often asymptomatic, and thus constitute a reservoir of infection (Nielsen et al., 1975). They may occasionally complain of vaginal discharge, but this may be attributable to concomitant vaginitis caused by Trichomonas

vaginalis (Robertson et al., 1980). The sites infected in the uncomplicated cases are the cervix (87-92 per cent), urethra (65-75 per cent), rectum (25-50 per cent), and oropharynx (2-5 per cent) (Young et al., 1979).

Oropharyngeal gonorrhoea refers to the infection of the pharynx resulting from transfer of organisms from the genitalia during fellatio or, less commonly cunnilingus (Robertson et al., 1980). Although there is a significant correlation between symptoms of pharyngitis and the practice of fellatio, the isolation of N. gonorrhoeae from the pharynx does not correlate with symptoms of pharyngitis (Wiesner et al., 1973). Symptoms are present in only about 20 per cent of cases (Stolz and Schuller, 1974), when the patient's complaint is sore throat, perhaps with referred pain in the ear.

The majority of patients (more than two-thirds) with anorectal gonorrhoea have no symptoms of infection (Catterall, 1962). In the male, anorectal gonorrhoea is invariably acquired through homosexual anal intercourse. Proctoscopic examination may show a normal appearance, or there may be either patchy or generalized erythema of the rectal mucosa with mucopus in the lumen of the anal canal and rectum. The anal canal, constructed of stratified cuboid or squamous epithelium, is not affected by the gonococcus (Robertson et al., 1980).

1.2 Local complications of gonorrhoea

These have been reviewed by Handsfield (1978) and Robertson et al. (1980). Pelvic inflammatory disease, including salpingitis, is the most important local complication of untreated gonococcal

infection; it is worthy of special mention since studies are now under way to characterize strains isolated from patients with this complication.

1.3 Disseminated gonococcal infection (DGI)

This uncommon complication, occurring in less than one per cent of cases, is usually seen in women and in homosexual men in whom the infection has been asymptomatic and untreated (Graber, Sanford and Ziff, 1960; Bayer, 1980). Dissemination may occur from any infected site and more often during or just after menstruation and in pregnancy.

Gonococcal strains associated with DGI are usually of the same auxotype (Arg⁻ Hyx⁻ Ura⁻), extremely sensitive to penicillin G, and resistant to the complement-dependent bactericidal action of normal human serum (Schoolnik, Buchanan and Holmes, 1976).

The clinical manifestations of DGI usually take the form of fever, rash, skin lesions and arthralgia or arthritis (Robertson et al., 1980).

Meningitis is an uncommon manifestation of DGI and is usually found associated with arthritis and dermatitis (Holmes, Counts and Beaty, 1971); gonococcal endocarditis is also a rare complication. The adult respiratory distress syndrome has also been associated with DGI (Markham, Vilseck and O'Donohue, 1976; Walters and Goldstein, 1980).

1.4 Gonorrhoea in infants and children under the age of puberty

Gonococcal ophthalmia neonatorum is a conjunctivitis with a purulent discharge which appears within 21 days of birth; it is a notifiable disease in the United Kingdom. The eyelids swell and pus collects in the conjunctival sac. Keratitis with corneal scarring and blindness may result if the condition is not treated (Robertson et al., 1980).

Acute gonococcal vulvo-vaginitis is uncommon in the United Kingdom nowadays (Robertson et al., 1980). Recently, the "resurgence of this old problem" was reported in France with three cases of gonococcal vulvo-vaginitis in school girls aged 5, 6 and 10 years (Boiron and Maleville, 1979; Maleville et al., 1979).

2. Host-parasite interactions and immunity

2.1 Interactions at mucosal surfaces

The healthy mucosal surfaces of the body abound with adherent commensal bacteria; the critical determinant of pathogenicity at these sites would seem to be the ability of the attached micro-organisms to penetrate the epithelial cell lining. Primary infection commonly occurs in the columnar epithelium. It has been shown that in patients with acute gonococcal urethritis, by the third day of infection, gonococci have penetrated to the subepithelial connective tissue (Harkness, 1948).

Since in nature N. gonorrhoeae is pathogenic only for humans, experimental infectivity studies are limited, and the lack of a

suitable model prevented further studies on gonococcal infection for a considerable time. There are experimental animal models that have been used to evaluate the pathogenicity of gonococcal strains, immunity, the immunogenicity of gonococcal cellular constituents, and candidate vaccines: the chimpanzee model, the guinea pig subcutaneous chamber, intracerebral infection in the mouse, gonococcal challenge of embryos obtained from vaccinated hens, and goat mastitis (World Health Organization, 1978).

Gonococci must adhere to mucosal surfaces in order to resist the shearing forces that result from urine and mucus flows. There is now abundant evidence that piliate gonococci from colony-types 1 or 2 attach more readily to human cells than do non-piliate gonococci from colony-types 3 or 4. Pili facilitate attachment to epithelial cells (Punsalang and Sawyer, 1973), to human cells in tissue culture (Swanson, 1973; Gubish et al., 1979), to human fallopian tubes in organ culture (Ward, Watt and Robertson, 1974), and to human sperm (James-Holmquest et al., 1974). Only piliate gonococci are capable of producing direct agglutination of human erythrocytes. Pili are at least one mediator of the attachment of piliate gonococci to human erythrocytes, since isolated pure pili are capable of causing haemagglutination. Moreover, antiserum to the homologous pilus type inhibits the haemagglutination produced by piliate gonococci or by isolated pure pili (Buchanan and Pearce, 1976).

The selectivity of the gonococcus as a pathogen for man could be explained by specific attachment to a receptor unique



to human cell membranes. However, to what extent pili promote attachment via specific receptors or non-specific mechanisms remains to be elucidated. Haemagglutination by piliated gonococci was not inhibited by 11 sugars including mannose, or by treatment of erythrocytes with trypsin or neuraminidase (Punsalang and Sawyer, 1973). The nature of the attachment is therefore quite distinct from the mannose-sensitive attachment in E. coli.

Non-specific mechanisms such as hydrophobic interaction may be involved in attachment; hydrophobic regions on pili could become attached by being embedded in the lipid interior of the host cell membranes. Heckels et al. (1976) suggested that the ability of pili to enhance attachment lies in overcoming the initial electrostatic repulsion between the gonococcus and the host cell; the overall negative surface charge on gonococci produces a significant barrier to attachment to host cells.

2.2 Invasion

Ward et al. (1974) reported the use of human fallopian tubes in organ culture as a model to study the mechanism of penetration. Electron microscopic studies of the infected fallopian tube confirmed that gonococci invade columnar epithelium. Subsequently, Watt, Ward and Robertson (1976) have shown that gonococci invade epithelial cells by phagocytosis (as opposed to invasion by erosion). It is thought that gonococci in the intercellular spaces are derived from the epithelial cells by a process of exocytosis. Multiplication of intra- and intercellular gonococci in the submucosa may result in the lateral spread of the infection (Watt and Ward, 1977).

A priori it would seem that the factor(s) responsible for the ability of gonococci to invade mucosal surfaces are expressed on the envelope of the organism. The gonococcal envelope has already been described; knowledge of the detailed molecular architecture of the gonococcal surface would be an important aid to understanding the role of surface components in gonococcal pathogenesis. Obviously, structures known to be involved in attachment to and invasion of mucosal surfaces or resistance to host defense mechanisms are potential candidates for use in a vaccine.

To invade mucosal surfaces and tissues successfully, a bacterium must both adhere to the surface and evade the host's defenses in the tissues. In addition to providing virulent gonococci with a means of attachment, pili are thought to confer resistance to phagocytosis (Punsalang and Sawyer, 1973). Various studies have demonstrated that virulent N. gonorrhoeae colony-types 1 and 2 attach to the surface of human polymorphonuclear leukocytes but resist ingestion (Thomas, Hill and Tyeryar, 1973; Thongthai and Sawyer, 1973; Ofek, Beachey and Bisno, 1974; Dilworth, Hendley and Mandell, 1975). In contrast, the avirulent colony-types 3 and 4 are readily ingested in the presence of serum (Gibbs and Roberts, 1975; Schiller, Friedman and Roberts, 1979). The failure of polymorphonuclear leukocytes to kill surface-attached gonococci was investigated and it appeared to be a consequence of the failure of these phagocytes to enclose the virulent gonococci within a phagosome (Densen and Mandell, 1978). The phagocytic vacuole thus plays a critical role in normal polymorpho-

nuclear bactericidal activity by providing a closed space in which the proper concentration of substances may be achieved to generate microbicidal activity.

As pili are rarely seen on gonococci in pus (Novotny et al., 1975), they may not be a critical virulence determinant in vivo. Various studies lend support to the belief that other components of the gonococcal outer membrane are involved in virulence. Salit and Gotschlich (1978) made the observation that piliate gonococci from transparent colonies are more virulent than piliate gonococci from opaque colonies in the chick embryo model. Moreover, strains of gonococci isolated from infected women at the time of menses give rise primarily to transparent colonies, whereas isolates obtained in mid-cycle yield predominantly opaque colonies (James and Swanson, 1978). The high frequency of secondary complications such as pelvic inflammatory disease observed at or near menses (Rees and Annels, 1969) suggests that transparent strains are more invasive. It is interesting that increased virulence in this case is associated with the absence of a protein(s) associated with colony-opacity. However, the importance of this phenotypically expressed change is not known, as it may be only a reflection of other changes which result in increased virulence of the gonococcus.

Another outer membrane component which promotes attachment to leukocytes (leukocyte association protein) may play an important role in pathogenesis (King and Swanson, 1978).

The ability of gonococci to invade has also been related to the acquisition by gonococci of a new major outer membrane protein, when transformed to resistance to serum bactericidal activity

(Hildebrandt et al., 1978). The relationship of this protein to the mechanism of resistance is not known.

The direct involvement of gonococcal lipopolysaccharide (LPS) in pathogenesis has been suggested recently by Gregg, Melly and McGee (1980). Gonococci were shown to damage the mucosa of human fallopian tubes in organ culture, producing characteristic pathologic features. Filter-sterilized supernatant fluid from these cultures damaged non-infected organ cultures in a similar fashion. The toxic activity was attributed to LPS which was detected in supernatant fluid; purified LPS, at concentrations much lower than those found in the toxic fluids, produced damage equivalent to that caused by supernatant fluid from infected organ culture. These studies also suggested that the toxic moiety of gonococcal LPS, as with endotoxins of other Gram-negative bacteria, is the lipid A portion of the molecule.

Culture filtrates of all four colony-types of N. gonorrhoeae contain a proteolytic enzyme which cleaves a susceptible proline-threonine bond in the hinge region of the heavy chain of human serum IgA 1 and human secretory IgA 1 (Plaut et al., 1975); the IgA 2 subclass is not affected. The purification of this enzyme from broth cultures of N. gonorrhoeae has been described by Blake and Swanson (1978). IgA 1 protease activity could also be demonstrated in vaginal washings obtained from most women with genital gonorrhoea (Blake, Holmes and Swanson, 1979). The importance of this protease in the virulence of N. gonorrhoeae would be influenced by the antigonococcal IgA subclasses present on mucosal surfaces. To date, the subclass involved in the local

antibody response to gonococcal infection is not known.

According to one concept, gonococcal pathogenicity may be based primarily on internal disorganization of human macrophages. Novotny et al. (1977) proposed that human gonococcal infection may have the following sequence. The host is infected with a mixture of free, non-multiplying gonococci, and multiplying gonococci in infectious units. Infectious units make contact with epithelial cells. The presence of multiplying gonococci is highly leukotaxic and this leads to mobilisation of pus cells and to infiltration of the mucosa and submucosa. All free gonococci are phagocytosed. Those phagocytosed by polymorphs are destroyed, whilst those taken up by mobilised mononuclear phagocytes interfere with the regulation of the cells and multiply. The multiplying gonococci become densely surrounded by the remnants of the host cell which is destroyed, and a new infectious unit is formed. The host cell remnants are utilised; the gonococci become less and less coated and are rephagocytosed, and the cycle is repeated.

2.3 Humoral antibody

Antibodies to N. gonorrhoeae have been detected by a variety of serological methods as discussed at p. 80 . The classes of immunoglobulin reactive with N. gonorrhoeae have, however, been largely defined by immunofluorescence studies. In one such study, Cohen (1967) noted that sera contained natural as well as induced antibodies reactive with N. gonorrhoeae; these antibodies were of the IgG, IgM and IgA classes.

Recent studies have attempted to elucidate the roles played

by the various antibody classes in infection. "Natural" gonococcal bactericidal antibody is present in the serum of most normal adults and may prevent the systemic dissemination of serum-sensitive strains of N. gonorrhoeae; it arises without obvious immunization or specific infection (Schoolnik, Buchanan and Holmes, 1976). Most strains isolated from patients with disseminated gonococcal infection are found to be serum-resistant.

It has been demonstrated that the "natural" gonococcal bactericidal activity of normal human sera for serum-sensitive strains of N. gonorrhoeae is a function of IgM, as determined by the bactericidal activity of immunoglobulin fractions purified from whole serum, its susceptibility to mercaptoethanol, and its inability to cross the placenta (Schoolnik, Ochs and Buchanan, 1979). This is consistent with other studies of serum bactericidal activity that have concluded that "natural" antibodies to the somatic antigens of Gram-negative bacteria are principally of the IgM class (Michael and Rosen, 1963). Serum bactericidal activity was also markedly diminished in patients with well characterized immunologic defects associated with low IgM serum concentrations (Schoolnik et al., 1979). The finding that gonococcal bactericidal activity of normal human serum is mediated by IgM and complement was more recently confirmed by Rice, McCormack and Kasper (1980). These bactericidal antibodies appear to be directed against lipopolysaccharides (Glynn and Ward, 1970; Rice and Kasper, 1977) and possibly proteins in the outer membrane (Tramont, Sadoff and Artenstein, 1974).

Natural IgG has been ascribed the role of heat-stable opsonin,

promoting phagocytosis of gonococci by human polymorphonuclear leukocytes (Schiller et al., 1979). Similarly, Jones et al. (1980) reached the conclusion that the opsonic activity of anti-pilus IgG is mediated via recognition by human polymorphonuclear leukocytes of the Fc fragment of IgG molecules bound to gonococcal pili.

McCutchan et al. (1978) examined the mechanism of resistance by certain strains of N. gonorrhoeae to normal human serum, whereby these organisms can cause disseminated infection. Gonococci resistant to killing by normal human serum were killed in large numbers by normal rabbit serum; however human cord serum containing maternal IgG, but no IgM or IgA, blocked rabbit serum as effectively as normal adult or matched maternal serum. Also, IgG fractions added to rabbit serum reduced killing, whereas IgM and IgA did not. McCutchan et al. (1978) concluded that normal human serum contains an IgG-blocking antibody which competes with natural bactericidal antibody in man and rabbits. They postulated that natural IgG binds to certain gonococci possessing the appropriate receptor and enables them to disseminate in the blood by protecting them from killing by bactericidal antibody and complement.

Rice, Nugent and Kasper (1980) examined the possible role of specific outer membrane antigens in the recognition of natural IgG-blocking antibodies by comparing the outer membranes of serum-sensitive and resistant gonococci. SDS-polyacrylamide gel electrophoresis revealed differences in the two major outer membrane proteins; those of the serum-resistant gonococci had molecular weights of 40 000 and 27 000, whereas the corresponding values

in the serum-sensitive gonococci were 36 000 and 31 000 daltons. These outer membrane protein antigens were subsequently used as absorbents for blocking IgG. The greater avidity of serum-resistant proteins for blocking IgG in normal serum suggested a possible mechanism of resistance to normal human sera of certain strains, such as those that cause disseminated gonococcal infection.

2.4 Local antibody response

Gonorrhoea is essentially an infection of mucosal surfaces. Thus, development of local antibody may be an important host response to this infection. Kearns et al. (1973a) detected IgA antibodies to the gonococcus in the urethral exudates of 29 of 35 men who were diagnosed as having gonococcal urethritis, and further demonstrated that these antibodies were chiefly secretory IgA. Similarly, in a study of women infected with N. gonorrhoeae, specific secretory IgA was shown to be present in cervicovaginal secretions (O'Reilly, Lee and Welch, 1976).

In a similar study, using indirect immunofluorescence, McMillan et al. (1979a) detected antigonococcal IgA in cervical secretions from 71 (95 per cent) of 75 infected women and in none of the secretions from 70 non-infected women. Antibodies of the IgG and IgM classes were also detected in 73 (97 per cent) and 29 (39 per cent) of 75 infected women, respectively. Only IgG antibody could be detected in 23 (33 per cent) of 70 non-infected women. Successful treatment resulted in a rapid decline in IgA antibody activity, but a more gradual decrease in IgG reactivity.

An examination of urethral exudates from 132 infected men showed IgA antibodies to be present in 129 (98 per cent), IgG in 119 (90 per cent), and IgM in 64 (49 per cent) (McMillan, McNeillage

and Young, 1979b). IgA and IgG antibodies to the gonococcus were found in the secretions of only one (2 per cent) and 13 (26 per cent) of 50 men with non-gonococcal urethritis, respectively. In patients who had no evidence of urethritis, no antibodies could be detected. IgA antibody appeared early in infection, followed by IgG slightly later. McMillan et al. (1979b) suggested that it is possible that the majority of IgG in the secretions is derived from non-specific transudation through inflamed mucous membranes; natural antibody of this class can be detected in the sera of non-infected patients (Cohen, 1967). In most cases, IgA and IgM antibodies to the gonococcus could not be detected in the secretions within two weeks of successful treatment for gonorrhoea; a more gradual decline in IgG reactivity was observed.

Antibodies reactive with N. gonorrhoeae in secretions on extra-genital surfaces have also been investigated (McMillan, Young and McNeillage, 1980). Antigonococcal antibodies of the IgA and IgG classes could be detected in only a small proportion of patients with ano-rectal gonorrhoea. An antibody response to infection in the oro-pharynx was found in most patients with infection of these surfaces.

2.5 Complement

Complement is a complex system of components which plays an important role in the inflammatory response and in immunologically mediated host defense. Interaction of bacteria with serum produces sequential activation of the terminal complement components (C3 through C9) via the classic (C1, C4 and C2) or

alternative pathways, or both. As a consequence of complement activation, phlogistic peptides are released, phagocytosis of foreign particles is enhanced, and bacteria are lysed (Ruddy, Gigli and Austen, 1972).

The frequent occurrence of N. meningitidis and N. gonorrhoeae bacteraemia in patients with deficiency of C6, C7 or C8 underlines the importance of complement in serum bactericidal activity (Petersen et al., 1979). Gonococcal serum bactericidal activity is primarily associated with and dependent on activation of the classic complement pathway. Although activation of the alternative pathway also occurs, it is expressed only after a latent period and may be antibody-dependent (Ingwer, Petersen and Brooks, 1978).

Schiller et al. (1979) studied the role of natural IgG and complement in the phagocytosis of N. gonorrhoeae colony-type 4 by human polymorphonuclear leukocytes. The requirement of fresh normal human serum for optimal phagocytosis, and the fixation of IgG and complement (C3) to the gonococcal cell surface suggested that both serum factors participate in the phagocytosis of these organisms. Although complement alone did not promote phagocytosis, it enhanced IgG-mediated ingestion. Studies using C2-deficient serum or serum chelators indicated that the alternative complement pathway participates in this reaction.

2.6 Cell-mediated immune response

Delayed hypersensitivity to N. gonorrhoeae antigens has been demonstrated in vivo in patients with gonococcal infections (Corbus and Corbus, 1941). More recent reports of in vitro lymphocyte activation in response to gonococcal antigens leave

no doubt as to the existence of a cell-mediated immune response in humans infected with N. gonorrhoeae (Kraus, Perkins and Geller, 1970; Esquenazi and Streitfield, 1973; Grimble and McIlmurray, 1973; Kearns et al., 1973b; Wyle, Rowlett and Blumenthal, 1977). These studies indicate that there is a systemic cellular immune response to gonococcal antigens in patients with uncomplicated gonococcal infections, it may have a functional role in preventing dissemination of the infection beyond the primary genital mucous membrane sites.

Although there are both humoral and cell-mediated immune responses to gonococcal infection, they appear to be inadequate to prevent reinfection. The short incubation period with rapid production of symptoms usually leads to the prompt treatment of infected males; this limits the time during which antigen can operate before removal by treatment. In women, gonorrhoea is frequently asymptomatic, so that the duration and degree of antigenic stimulation are greater (World Health Organization, 1973). Immunological mechanisms are probably responsible in part for limiting the spread of gonococcal infection beyond its primary site. Buchanan et al. (1980) obtained data suggesting that an episode of gonococcal pelvic inflammatory disease (salpingitis) produces some immunity to repeated episodes of salpingitis with the same protein I serotype, while not preventing reinfection with the same protein I serotype. The immune response to protein I antigen may thus provide serotype-specific protection against gonococcal salpingitis.

LABORATORY PROCEDURES FOR THE DIAGNOSIS OF GONORRHOEA

Microbiological tests are mandatory in making a diagnosis of gonorrhoea. Because of the short incubation period and high infectivity, rapid diagnosis followed by immediate treatment is important in the control of infection within the community.

Neisseria gonorrhoeae is a very fastidious organism and very careful procedures are necessary for the collection of specimens and their transport to the laboratory for culture and investigation. Ideally, the patient is seen at a clinic with an adjacent or closely-sited laboratory.

1. Immediate diagnosis

Immediate diagnosis is extremely important as it enables appropriate treatment to be given without delay.

1.1 Gram-staining

Gram-staining of genital secretions remains the only widely accepted routine procedure for making an "on-the-spot" diagnosis of gonococcal infection (Young, 1981).

An unequivocally positive or negative Gram-stained smear of urethral discharge from a man provides an immediate differential diagnosis between gonococcal and non-gonococcal urethritis in 85 per cent of male patients (Jacobs and Kraus, 1975). More recently, the sensitivity of this method was reported to be 99.1 per cent in a group of 113 symptomatic male patients (Luciano and Grubin, 1980).

For women, Gram-staining of smears of cervical and urethral secretions is less reliable, detecting only 55 to 65 per cent of patients who subsequently give positive cultures (Barlow et al., 1976; Chipperfield and Catterall, 1976; Evans, 1976). More recently, Barlow and Phillips (1978) reported that Gram-staining of urethral samples made no significant contribution to the diagnosis of gonorrhoea in women. A similar conclusion was reached by Thin and Shaw (1979).

Gram-staining has no place in the diagnosis of pharyngeal infection in either sex, and rectal smears are rarely carried out as a routine (Young, 1981). In homosexual men, routine examination of Gram-stained smears of rectal material obtained by proctoscopy was found to be of limited diagnostic value (Deheragoda, 1977; McMillan and Young, 1979); the value of this procedure may be increased if pus or mucopus can be collected for examination. Thin and Shaw (1979) reported a low yield from rectal smears in women. However, Barlow and Phillips (1978) considered Gram-staining of material taken from the rectum under direct vision to be of some value in women who are known gonorrhoea contacts: of 607 episodes of gonorrhoea studied, a positive rectal smear was the only test positive in three patients.

1.2 Immediate immunofluorescence

Fluorescent antibody (FA) staining of secretions direct from the patient has not provided a more sensitive and reliable routine diagnostic method than Gram-staining.

Results with the immediate FA test do not appear to be consistent when compared with Gram-staining and culture. Hare

(1974) made a comparative assessment of microbiological methods for the diagnosis of gonorrhoea in women and examined results from several published trials. For standardization, the most sensitive method in each trial was expressed as 100, and other methods as percentages of this. In four of eleven studies the direct FA test was found to be the most sensitive method, but in three it scored less than 60 per cent in comparison with other methods. Hare (1974) concluded that the FA test seems to be unsuitable for routine work, but is useful in the investigation of special cases and in research.

Danielsson and Forsum (1975) assessed the methodology of immunofluorescence applied to the diagnosis of neisserial infections and concluded that the immediate FA test requires highly skilled staff and is too laborious for routine use. Nevertheless, like Hare (1974), they considered that it could be useful on special occasions, for example when the patient has been given antibiotics prior to testing and it is anticipated that culture will be negative.

In support of these opinions, Tronca et al. (1974) found the direct FA test a valuable tool in the study of the pathogenesis of DGI and in the rapid diagnosis of this disease. Direct FA staining of exudate from skin lesions of patients with DGI was found to be significantly more sensitive than either culture or Gram-staining. Nineteen (66 per cent) of 29 specimens of exudate from skin lesions examined simultaneously by all three methods were positive by at least one method: in 18 (62 per cent) of the cases, gonococci were demonstrated by the FA test, in three (10 per cent) by culture, and in four (14 per cent) by Gram-staining. Great care must be taken in interpreting results obtained with exudates from skin lesions, since specimens from two patients with staphylococcal pyoderma contained

rounded organisms that stained non-specifically but did not resemble the bean-shaped diplococci typical of N. gonorrhoeae.

FA testing of peripheral blood buffy coat smears also appeared to be a useful adjunct to routine blood cultures for detecting gonococcal bacteraemia, particularly in patients who had received recent antibiotic treatment (Tronca et al., 1974).

2. Culture

2.1 Selective media

The importance of selective media is now established beyond doubt; over 70 per cent of laboratories in England and Wales now use some form of selective medium (Adler, 1978). The mechanism whereby a selective medium improves the cultural diagnosis of gonorrhoea is probably twofold: firstly it prevents the growth of other flora in the sample from overgrowing gonococci and masking their presence, secondly it prevents the growth of several bacterial species that are capable of inhibiting the growth of gonococci (Kraus et al., 1976). Shtibel (1976) noted that N. meningitidis, Staphylococcus epidermidis, Corynebacterium species, Staphylococcus aureus, group A streptococci, and N. subflava could all inhibit the growth of N. gonorrhoeae. Inhibition of gonococci by components of the normal endocervical flora was also noted by Saigh, Sanders and Sanders (1978) who considered it possible that such antagonistic actions towards gonococci might prevent the establishment of infection. Certain strains of Candida albicans also produce a substance that is inhibitory for some strains of N. gonorrhoeae in vitro (Hipp et al., 1974; Hipp et al., 1975).

The first selective medium formulated to grow only gonococci and meningococci was developed by Thayer and Martin (1964, 1966). This medium which contains the antibiotics, vancomycin, colistin and nystatin, has been criticized for the following reasons:

3 per cent (Reyn, 1969; Reyn and Bentzon, 1972) to 10 per cent (Brorson et al., 1973) of gonococcal strains are inhibited by vancomycin at the concentration used; it does not prevent spoilage of cultures by overgrowth with Proteus species; nystatin at the concentration used is not an effective inhibitor for C. albicans; and growth of gonococci is slow and colonies are small.

Superior culture results were obtained by Martin, Armstrong and Smith (1974) with a modified Thayer Martin (MTM) medium. MTM medium differed from TM medium in containing an increased agar concentration (2 per cent), additional glucose (0.25 per cent) and trimethoprim (5 µg/ml). When 328 cervical specimens were cultured in both media, 123 (37.5 per cent) were positive on MTM whereas 110 (33.5 per cent) were positive on TM medium. Also, MTM completely eliminated spreading growth by Proteus, whereas 19 (5.8 per cent) of the cultures on TM medium were overgrown with spreading Proteus.

A further modification of MTM medium was made by Martin and Lewis (1977). In an attempt to solve the problem of yeast overgrowth and inhibition, anisomycin was substituted for nystatin. Also, the concentration of vancomycin was increased in order to inhibit Gram-positive bacteria. Martin Lewis medium was reported to have a longer shelf life than media containing nystatin. Recently, Smeltzer, Curran and Lossick (1979) failed to confirm a report (Moyer and Parsons, 1977) on the enhanced recovery of gonococci on Martin Lewis medium. However, the visualization of plates for the presence or absence of gonococcal colonies was somewhat easier owing to the enhanced inhibition of yeasts

and Gram-positive organisms on Martin Lewis medium (Smeltzer et al., 1979).

In 1973, a new selective medium designated New York City (NYC) medium was described (Faur et al., 1973a, 1973b); it provided luxuriant growth of pathogenic neisseriae after only 24 hours incubation. NYC medium essentially consists of a proteose peptone-corn starch agar buffered base to which is added a haemoglobin solution prepared from fresh horse erythrocytes, horse plasma, yeast dialysate, glucose and vancomycin, colistin, amphotericin B and trimethoprim lactate.

As originally described by Faur et al. (1973a, 1973b), NYC medium is inconvenient for many service laboratories to prepare; the modified New York City (MNYC) medium described by Young (1978a) may be more suitable for routine use. The modified medium is much simpler to prepare than the original since it uses a commercially available gonococcal base. The other major simplification in MNYC medium is the use of completely lysed whole blood in place of plasma and haemoglobin solution made from fresh horse erythrocytes.

In MNYC medium, lincomycin is used in place of vancomycin since it is less inhibitory to gonococci (Young, 1978a). Amphotericin B is used in place of nystatin in both media because it is more inhibitory to yeasts. Yeast dialysate is also used in both media since yeast extract may contain macromolecular substances that have some toxic effect on gonococci; these are eliminated by dialysis.

Although MNYC medium was not compared directly with the

original NYC medium, both media resulted in similar improvements when they were compared with TM medium in separate trials (Faur et al., 1973b; Young, 1978a). In comparison with TM medium, NYC and MNYC media improve the overall isolation rate and enable a larger percentage of isolates to be identified at 24 hours. These findings are of great value in allowing rapid isolation and identification; in many of the identification methods described later, young 18 to 24 hour colonies are preferable to 48 hour ones.

More recently, MNYC medium was found to be superior to a chocolate-agar medium containing nystatin, colistin and vancomycin (Svarva and Moeland, 1979). In a separate trial, MNYC and Martin Lewis media were found to be comparable in their ability to grow gonococci from clinical material (Granato, Paepke and Weiner, 1980). However, it was suggested that MNYC medium may have considerable application since it is capable of supporting the growth of pathogenic neisseriae, Mycoplasma pneumoniae, and urogenital mycoplasmas. It should be noted that evidence was presented for the growth of only M. pneumoniae on MNYC medium (Granato, Poe and Weiner, 1980), whereas it has been shown that NYC medium can readily support the growth of Ureaplasma urealyticum in addition to large-colony mycoplasmas (Faur et al., 1974).

2.2 Combination of a selective and non-selective medium

A significant proportion of clinical isolates of N. gonorrhoeae have been shown to contain mutations which result in a markedly increased susceptibility to a variety of antibiotics (Eisenstein and Sparling, 1978). These findings will lend support to the

earlier suggestion (Reyn, 1969) that ideally a combination of selective and non-selective medium should be used. Such a procedure is too time consuming, technically demanding, and not cost-effective for routine use, and in practice less than 10 per cent of clinics in England and Wales employ such a routine (Adler, 1978).

The recommendation of Jephcott and Rashid (1978) that a non-selective medium should be included in the subsequent examination of contacts of infected patients if their initial tests are negative is a more practical approach.

In Edinburgh where the selective medium contains lincomycin, colistin, amphotericin B and trimethoprim, only 1 per cent of cases in women gave typical positive Gram-stained smears which were not confirmed by culture (Young et al., 1979). In London and Sheffield where a selective medium containing vancomycin, colistin, nystatin and trimethoprim is used, the corresponding figures were 3 and 1.8 per cent respectively (Barlow et al., 1976; Jephcott and Rashid, 1978). Therefore, in several regions of the United Kingdom, sensitivity of gonococci to antibiotics in selective media does not appear to be a significant problem at present.

2.3 Transport and culture systems

When direct plating and immediate incubation is impracticable, several transport and culture systems are now available in addition to the conventional non-nutrient transport media such as Stuart's (1946), or Amies' modification of Stuart's medium (Amies, 1967).

These systems utilise a selective medium usually present in a small chamber containing CO_2 .

The first combined transport and culture system was described by Martin and Lester (1971) and designated Transgrow. It consists of a flat-faced bottle containing a modified Thayer-Martin medium and a carbon dioxide enriched atmosphere. Unfortunately, condensation on the glass produced on incubation may render visualization of the surface of the medium impossible, thus necessitating blind sampling for identification of growth of gonococci; even identification by the delayed fluorescent-antibody method has to be applied blindly (Jephcott, Morton and Turner, 1974).

The problems associated with Transgrow are overcome in the Jembec/Neigon system which is based on the biological environment chamber described by Martin and Jackson (1975). It consists of a rectangular culture plate containing a selective medium (usually modified Thayer-Martin medium). The plate incorporates a small recess into which a CO_2 -generating tablet is placed at the time of inoculation. The moisture of the plate ensures that CO_2 is generated owing to the reaction between the citric acid and sodium bicarbonate in the tablet. The closed plate is placed into a plastic bag which is sealed to make it gas-tight. This system offers easy direct access to the surface of the culture medium, so that suspect colonies can be further identified. In a comparison of Amies' medium with the Jembec/Neigon system, Jephcott, Bhattacharyya and Jackson (1976) obtained comparable results in terms of yield of positive cultures, but they found

that the latter system allowed more rapid diagnosis.

It should be pointed out that systems with slight modifications to those described are presently commercially available. When two of these systems (Bio-Bag and Gono-Pak) were compared with the Jembec/Neigon system, the differences obtained were not statistically significant (Carlson et al., 1980).

Dehydrated media have also been incorporated into kit-form (Microcult GC). These kits offer the advantage of a long shelf life and may be particularly useful in areas or countries which lack laboratory facilities (Willcox and John, 1976; Unsworth, Talsania and Phillips, 1979).

Faur et al. (1977) evaluated the recovery of N. gonorrhoeae with Transgrow and with NYC medium contained in plates and in a biological environment chamber. Their results suggested that the NYC-chamber system is an effective method for the handling, transport and culture of N. gonorrhoeae if the delay in transport and incubation does not exceed 24 hours.

The most suitable transport and culture system must take account of constraints imposed in individual localities. In general, direct plating seems to be the system that most consistently provides the best results for most specimens. The Jembec/Neigon system has the advantage over conventional non-nutritive transport media of providing a more rapid identification, and over the other transport and culture systems of allowing ready identification and antibiotic sensitivity testing of isolates. The Microcult system has the unique advantage of a very long shelf life. In a review of these systems, Riccardi and Felman (1979) suggested

that the Jembec/Neigon system offers a good choice where a complete laboratory is not available. While future trials will clarify the clinical and microbiological value and limitations of such systems, their cost may limit their widespread use.

3. Identification of isolates

After 24 hours of incubation, the plates are examined and the identity of suspected gonococcal colonies may be confirmed by various methods.

3.1 The cytochrome oxidase test

This is a useful screening test since all neisseriae are oxidase positive, whereas many other organisms such as coliforms, staphylococci, and lactobacilli are negative. The test is conveniently carried out by touching the colony with a cotton swab soaked in a one per cent (w/v) solution of tetramethyl-p-phenylenediamine dihydrochloride. A positive reaction is indicated by the colony turning purple within 5 to 15 seconds.

3.2 Gram-stained smear from colony

A presumptive diagnosis of gonorrhoea, from material taken from a genital site, can be made on the basis of oxidase-positive Gram-negative diplococci growing on selective medium. However, because the various members of the genus Neisseria are indistinguishable by microscopy, further identification is necessary to provide a precise diagnosis.

3.3 Delayed immunofluorescence

In contrast to the immediate immunofluorescence test described earlier, the immunofluorescence staining of a smear of suspect organisms obtained from the primary isolation plate is termed the delayed (FA) method. Provided that the test is well controlled, delayed immunofluorescence provides a rapid and reliable confirmation of the gonococcus in the majority of instances (Lind, 1975). While cross-reaction with meningococci has been largely eliminated by absorption of the conjugate with strains of N. meningitidis, the delayed FA identification is not recommended for throat cultures (Lind, 1975).

Chemical and immunological studies have shown that the R-type lipopolysaccharide (LPS) is a common antigen of N. gonorrhoeae (Perry et al., 1977; Diena et al., 1978). Hens immunized with gonococcal LPS produce antibodies to this outer membrane component (Wallace et al., 1978). In an attempt to improve the FA method, Ashton et al. (1979) prepared a fluorescent antibody reagent with sera obtained from hens immunized with gonococcal R-type LPS. The anti-LPS conjugate was absorbed with formalin-treated cells of N. meningitidis. The anti-LPS conjugate was compared with Difco Laboratories fluorescent antibody conjugate for staining of N. gonorrhoeae; they respectively confirmed the identity of 98.8 per cent and 98.6 per cent of the gonococcal cultures. The conjugate did not significantly stain cultures of N. meningitidis, N. lactamica, non-pathogenic neisseriae, or other Gram-negative bacteria. The use of anti-LPS conjugate does not appear to have

improved the delayed FA method. Anti-LPS conjugate absorbed with R-type LPS failed to stain N. gonorrhoeae, whereas similar treatment of the Difco conjugate had no effect on its ability to stain gonococci. This suggests that the majority of fluorescein-labelled antibody present in the Difco conjugate is directed to gonococcal cell-surface components other than LPS (Ashton et al., 1979).

3.4 Coagglutination

This method described by Danielsson and Kronvall (1974) is a rapid slide agglutination test which uses protein A-producing staphylococci with rabbit anti-gonococcal antibodies, bound by their Fc portions to the protein A. On mixing this reagent (available commercially as the Phadebact gonococcus test) with gonococci, a readily visible agglutination is produced. This reaction is compared with a control in which the staphylococci have been coated with non-immune rabbit immunoglobulin. For positive tests to be readable, there must be an easily distinguishable difference between the test and control reaction. Experience with the test is necessary to distinguish true agglutination and granular or auto-agglutination reactions (Barnham and Glynn, 1978). Inconclusive results can be converted to clearly positive or negative reactions by adding trypsin to the test system (Menck, 1976), or by heating the suspension of test organisms to 80 to 100°C for at least 20 minutes (Lewis and Martin, 1980).

Barnham and Glynn (1978) found that 98 per cent of 140 strains of gonococci gave positive reactions. However, the discrimination between N. gonorrhoeae and other Neisseria species

was poor. These findings differ from the results of Menck (1976) who found the test useful to distinguish between different Neisseria species. More recently, complete agreement was achieved with both the Phadebact and sugar utilization (Minitex system) tests for 236 isolates of N. gonorrhoeae (Hampton, Stallings and Wasilauskas, 1979). Of 78 non-gonococcal isolates tested with Phadebact, two isolates gave false positive results, whereas one isolate was reported as non-interpretable. In a separate evaluation of this method, the Phadebact gonococcus test correctly identified 97.8 per cent of 275 gonococcal isolates and gave a false positive result in 6 per cent of 33 other clinical isolates (Lim and Wall, 1980). However, the test was negative with all 29 laboratory strains which included not only other species of Neisseria, but also bacteria commonly found in sites cultured for N. gonorrhoeae.

The coagglutination test had been found to be reliable, rapid and simple to perform; it was judged as a good adjunct to the carbohydrate utilization test (Olcén, Danielsson and Kjellander, 1978). The more recent comparative evaluations of the various methods for the confirmatory identification of N. gonorrhoeae suggest that the slide coagglutination test which is highly sensitive, specific, rapid and simple to perform offers a valuable alternative to the other methods. Lewis and Martin (1980), in their evaluation of the Phadebact gonococcus test in comparison with the carbohydrate utilization and delayed FA tests, found the three methods to identify respectively 97.1, 93.1 and 98.7 per cent of 235 clinical isolates as gonococci. The test was found to be highly specific, showing no cross

reaction with 55 other Neisseria species or with 50 miscellaneous organisms.

This method appears very promising and deserves further evaluation, taking into account individual patients and the various sites examined.

3.5 Agglutination with anti-gonococcal lipopolysaccharide hen serum

Wallace et al. (1978) described a simple slide agglutination test for the identification of gonococci using antisera prepared in hens to R-type gonococcal LPS. Anti-LPS hen serum proved highly efficient for confirming the identity of gonococci in both primary and secondary cultures, and showed no agglutination with other neisseriae including N. meningitidis. This apparently sensitive, specific, and simple slide test requires further investigation.

3.6 Lectin slide agglutination test

A lectin slide agglutination test has been developed for the confirmatory identification of N. gonorrhoeae (Schaefer, Keller and Doyle, 1979). With wheat germ lectin as an agglutinin, 164 of 165 clinical isolates of N. gonorrhoeae gave a positive reaction within 6 to 8 minutes. Four gonococcal isolates, even though negative by the delayed fluorescent antibody method, gave strong positive reactions with the wheat germ lectin. Of the other neisseriae tested by this method, only one meningococcal strain belonging to the serogroup X gave a false positive reaction. This novel method provides a simple, rapid, and inexpensive means

for the confirmatory identification of N. gonorrhoeae, and should therefore be further evaluated.

3.7 Carbohydrate utilization

Carbohydrate utilization tests are necessary to establish the identity of Neisseria species which differ in their ability to utilise various sugars. Gonococci produce acid from glucose only, whereas meningococci produce acid from glucose and maltose, and N. lactamica utilises lactose in addition to glucose and maltose (Young, 1978b). These tests are also necessary to identify isolates giving negative or equivocal results in other tests, and provide a means of identifying gonococci antigenically distinct from those used in the production of anti-gonococcal antibodies for immunological tests. Carbohydrate utilization tests are basically of two types, conventional and rapid.

In conventional tests for carbohydrate utilization, a solid medium containing the appropriate carbohydrate and pH indicator is inoculated with the test organism. Although widely used, these tests are unsuitable since a positive reaction is dependent on adequate growth of the test organism which may take up to 72 hours (Pollock, 1976).

In the rapid carbohydrate utilization test (RCUT), pre-formed enzyme is measured by adding a suspension of the overnight growth of the suspect organism to a buffered, non-nutrient solution containing the sugar to be tested and a pH indicator (Young, Paterson and McDonald, 1976). Since the test measures pre-formed enzyme, it has the advantage of being independent of growth. It therefore presents no problems with fastidious strains since

they can be grown on a suitable medium first. Yong and Prytula (1978) described a modified RCUT which utilised both pre-formed enzymes and enzymes formed by the bacteria as a result of growth in a super-enriched medium. Because of the combined action of two sources of enzymes, this method is probably suitable for very small inocula. However, it is unlikely that it could be used to characterize isolates directly from primary isolation cultures because of possible contamination by other bacteria.

3.8 Antibiotic sensitivity tests

Once the gonococcus has been fully identified, antibiotic sensitivity tests are carried out. These are retrospective since the majority of patients will already have been treated on the basis of Gram-stained smear results. Nevertheless, they are important for epidemiological purposes (Jackson and Jephcott, 1976) and in planning rational therapy for use in the geographical area concerned.

Most laboratories carry out initial screening against several antibiotics using a disc method. The minimum inhibitory concentration (MIC) of any antibiotic to which the strain shows decreased sensitivity can then be determined by testing the ability of the strain to grow on medium containing a series of antibiotic concentrations. The lowest concentration of antibiotic which inhibits growth is taken as the MIC.

The majority of reports define a less sensitive (or relatively resistant) strain as one requiring 0.125 µg/ml or more of penicillin for inhibition (Wilkinson, 1977).

The recent discovery of strains of gonococci resistant to

penicillin because of penicillinase (beta-lactamase) production has increased the importance of laboratory antibiotic sensitivity tests (Wilkinson, 1977).

All strains with decreased sensitivity to penicillin ($MIC \geq 0.125 \mu\text{g/ml}$) should be tested for penicillinase production by one of the several tests now available (World Health Organization, 1978). One test depends upon the hydrolysis, by penicillinase, of the beta-lactam ring of a chromogenic cephalosporin substrate, giving rise to a coloured product (O'Callaghan *et al.*, 1972). Other tests depend upon the bacterial breakdown of penicillin to penicilloic acid, which is detected by the ability of penicilloic acid to dissociate a starch-iodine complex (Odugbemy, Hafiz and McEntegart, 1977) or by a pH indicator system (Phillips, Aller and Cohen, 1976). The latter method can be easily carried out alongside the rapid carbohydrate utilization test as part of the routine identification procedure, enabling all isolates to be tested, many directly from the primary isolation plate (Young, 1978c).

4. Non-cultural methods

4.1 Serological methods for the detection of anti-gonococcal antibodies

The gonococcal complement fixation test (GCFT) is the only test which has been used to any extent in routine diagnosis. Magnusson and Kjellander (1965) found that their GCFT procedure, using as antigen a suspension of 20 strains of heat-treated gonococci, had a high specificity and was a worthwhile complementary

tool for the diagnosis of uncomplicated or complicated gonorrhoea. However, there were cross reactions in patients with meningococcal infection or chronic bronchitis. Watt, Ward and Glynn (1971) compared serological tests comprising complement fixation, agglutination, and flocculation, and found the level of false positive results in all tests too high to permit their use for routine screening. The diagnostic value of a GCFT with N. gonorrhoeae strain 9 was recently investigated by Young, Henrichsen and McMillan (1980); their results agree favorably with those of previous investigators. From a consideration of the data available, this test is not recommended for the detection or exclusion of uncomplicated gonococcal infection (Low and Young, 1979).

Indirect immunofluorescent-antibody methods have been used for the detection of gonococcal antibody in serum. Gonococcal antibodies could be detected in the sera of almost 90 per cent of patients with gonococcal arthritis (Hess, Hunter and Ziff, 1965). Although minimal cross-reactivity with meningococci was demonstrated by these workers, Peacock et al. (1964) reported that reactivity, at a titre of ≥ 20 , could be obtained in this test with 64 per cent of sera from meningococcal carriers.

N. gonorrhoeae strain 9 is considered to possess antigenic features that might be quantitatively or qualitatively specific to gonococci (O'Reilly, Welch and Kellogg, 1973). This strain was used as antigen in the evaluation of the indirect immunofluorescent method (O'Reilly et al., 1973; Welch and O'Reilly, 1973). Using a polyvalent fluorescein-conjugated antiserum, only four per cent of non-infected women had serum antigenococcal

antibody at a titre of ≥ 16 ; almost 80 per cent of women and 60 per cent of men with untreated gonorrhoea had antibody at this titre. The same antigen was used by Reddick (1975) who found a false positive rate of 15 per cent in pregnant women attending an antenatal public health clinic. The sensitivity could not be assessed, as only seven of 600 women had a gonococcal infection. The test was considered of little value in screening low-risk populations. In a separate evaluation of the test, a subculture of a primary isolate of N. gonorrhoeae was used as antigen (Wilkinson, 1975). Although the specificity of the test was comparable to that reported by Welch and O'Reilly (1973), its sensitivity was considerably lower.

Encouraging results were reported by Gaafar and D'Arcangelis (1976) who used as antigen strains of N. gonorrhoeae possessing a species-specific antigen designated L-antigen, and an anti-human IgG conjugate. Sera from 95 per cent of infected women and 87 per cent of infected men, but from only 1.4 per cent of non-infected individuals, were reactive. When the test was evaluated further (Gaafar, 1976) in screening high and low risk groups of patients, the sensitivity was reduced to 81 per cent in low risk, and 75 per cent in high risk groups of women. The specificity in the low risk group was just over 90 per cent.

Indirect immunofluorescence has also been used for the detection of gonococcal antibody in genital secretions. When secretions from the male urethra (McMillan *et al.*, 1979b) and from the uterine cervix (McMillan *et al.*, 1979a) were examined, antibody reactive with N. gonorrhoeae was detected in the secretions of 98 per cent of 132 infected men and in 97 per cent of 75 infected

women. All the men and 95 per cent of the women in whom antibody was detected had antigenococcal IgA. Successful treatment was associated with a rapid decline of specific IgA activity, suggesting that the value of such a test would not be limited greatly by detecting adequately treated patients.

Radioimmunoassay, a highly sensitive test system, has been used to detect gonococcal antibodies in serum. Buchanan et al. (1973) found significant levels of antibody against pilus antigen in the sera of about half of the men and 86 per cent of the women with gonorrhoea. Using an ^{125}I -labelled pilus antigen, Oates et al. (1977) detected antibody in the serum of 86 per cent of women with gonorrhoea and in 13 per cent of non-infected women. In a separate study, using an outer membrane extract, gonococcal antibodies were detected in the sera of all infected women (Luoma, Cross and Rudbach, 1975); however 22 per cent of non-infected women also had detectable antibody. More data are required before conclusions can be drawn about the value of radioimmunoassay in the serodiagnosis of gonorrhoea.

The first report on the use of an enzyme-linked immunosorbent assay (ELISA) in the serological diagnosis of gonorrhoea was made by Glynn and Ison (1978). The antigen used was an outer membrane protein prepared from a clinical isolate of N. gonorrhoeae. In a similar assay, gonococcal pilus protein was used as antigen (Young and Low, 1981). At present, these tests are hampered by lack of both sensitivity and specificity; the positivity rate in infected women being respectively approximately 55 per cent (Glynn and Ison, 1978), and 57 per cent (Young and Low, 1981),

while the corresponding reactivity in controls was 11 per cent and 6 per cent.

An enzyme-linked immunosorbent assay was used to detect antibody in rabbits immunized with outer membrane complex antigens extracted from N. gonorrhoeae (Brodeur, Ashton and Diena, 1978). Owing to its specificity as well as high sensitivity, accuracy, and reproducibility, the authors suggested that the enzyme-linked immunosorbent assay may provide the serological test required to detect and quantify specific antigenococcal antibody or gonococcal antigens present in secretions of humans.

Other tests such as flocculation tests (Lee and Schmale, 1970; Wallace et al., 1970; Watt, Ward and Glynn, 1971), precipitin tests (Reising and Kellogg, 1965; Chacko and Nair, 1969), and passive haemagglutination tests (Ward and Glynn, 1972; Fletcher, Miller and Nicol, 1973) have been described for the serodiagnosis of gonorrhoea; these appear to be of little value owing to low sensitivity and/or specificity. Counterimmunoelectrophoresis (Kwapinski, Kwapinski and Webb, 1978) and crossed immunoelectrophoresis (Salton, Friedman-Kien and Urban, 1978) have also been suggested as potential serodiagnostic methods.

4.2 Detection of gonococcal components

A method of detecting gonococcal antigens by solid-phase radioimmunoassay with radioactively labelled antibody was described by Thornley et al. (1979a); urine sediments were tested by this method. When sediments from samples of urine from male patients with gonorrhoea were tested, 31 (74 per cent) of 42 gave positive results, clearly distinguishing them from urine samples

from men with non-gonococcal urethritis, none of which was positive. Ten of 14 (71.4 per cent) urine sediments from urine samples from women with gonorrhoea gave positive results, as did 3 of 18 sediments from urine samples from women patients without gonorrhoea. Therefore gonococcal antigens can be detected in urine by radio-immunoassay; the method could be useful in diagnosis if, after refinement, its sensitivity and specificity were to be increased. The authors suggested that the use of purified antigens to raise antibody, and affinity purification of antibody should improve greatly the sensitivity of the assay.

A major problem in this assay was its inhibition by the supernatants of two-thirds of urine samples from male patients with either gonorrhoea or non-gonococcal urethritis (Thornley et al., 1979b). The inhibition was reduced or completely abolished by the addition of soybean trypsin inhibitor (STI); STI-sensitive inhibition was thought to be due to proteolytic enzymes, probably from pus cells. Their inhibitory effect was shown to be due to their action on gonococcal antigens and not on antibodies in the assay system. Some supernatants contained other inhibitors unaffected by STI. The authors suggested that STI-treated sediments could, therefore, be used in an assay designed to detect gonococcal antigens.

Transformation of gonococci, as a means of detecting gonococcal deoxyribonucleic acid (DNA) in clinical specimens, was investigated by Janik, Juni and Heym (1976). The detection of gonococcal DNA was based on the transformation of auxotrophic mutants (indicator strains) of N. gonorrhoeae to prototrophy by crude DNA preparations

obtained from clinical specimens. The recipient auxotrophic mutants included in this trial were mutant 488, a uracil and arginine auxotroph, and five other proline auxotrophs. The greatest specificity was achieved using mutant 488 in the transformation assay. DNA preparations from only a few other neisseriae were able to transform the proline auxotrophs, and none of such preparations were able to transform mutant 488 under the standard transformation assay conditions. Proteus, Candida albicans and other clinically isolated Gram-positive and Gram-negative organisms gave negative results when used as donors. A minimum of approximately 50 colony-forming-units of donor gonococci was required for a positive transformation assay, thus reflecting the high sensitivity of the assay. Janik et al. (1976) obtained a positive transformation assay result with 90 specimens confirmed by the conventional identification methods. Nevertheless, 39 clinical specimens from which N. gonorrhoeae could not be isolated gave a positive assay. However, the positive results obtained with these 39 specimens were shown to correlate with the clinical findings, symptoms, and histories of the patients from whom the specimens were obtained.

In a separate trial, this method was evaluated in a clinical laboratory setting and identified 97.1 per cent of 71 clinical isolates of N. gonorrhoeae (Bawdon, Juni and Britt, 1977). The two gonococcal isolates which gave a negative assay result were found to have similar auxotrophic requirements to those of the indicator strain 488. Nevertheless, the results confirmed the high degree of specificity of the assay using this particular

indicator strain.

As auxotrophy is a common feature among gonococci (Crawford, 1978), a transformation assay using a temperature-sensitive mutant of N. gonorrhoeae strain tsA-1 as indicator strain was recently described (Zubrzycki and Weinberger, 1980). The results obtained in this trial suggest that the use of a temperature-sensitive mutant as indicator strain offers an attractive alternative to the use of auxotrophic strains; the transformation assay in this case depends merely on the inability of the recipient strain of N. gonorrhoeae to grow at 37°C unless transformed.

These studies illustrate the potential value of a genetic transformation assay in the detection/identification of N. gonorrhoeae; this assay system therefore deserves further investigation.

An assay for endotoxin resulted from the finding by Levin and Bang (1964) that a lysate of washed amoebocytes of the horseshoe crab Limulus polyphemus formed a gel in the presence of minute amounts of endotoxin elaborated by Gram-negative bacteria. Following the demonstration by Rice and Kasper (1977) of the sensitivity of the Limulus endotoxin assay for components of N. gonorrhoeae, the system has been shown to be of value in the rapid presumptive diagnosis of gonococcal urethritis in men (Spagna, Prior and Perkins, 1979). Endotoxin was detected in urethral exudates from 73 patients with culture-positive gonococcal urethritis, while negative results were obtained from 26 out of 27 patients with culture-negative urethral specimens. Consequently, the authors suggested that the method may also be of value in identifying cases of non-gonococcal urethritis. Following this

encouraging report, the work on the Limulus lysate assay was extended in a comparative evaluation of the tube and microdilution Limulus lysate methods for rapid presumptive diagnosis of gonococcal urethritis in men (Prior and Spagna, 1980). This modification of the standard Limulus lysate tube assay in which microdilution plastic plates were used to quantify endotoxin had already been described in a previous report (Prior and Spagna, 1979). The microdilution method has the added advantage of requiring a smaller volume of lysate, and of abbreviating the time of performance. It also provides reliable results over a wide dilution range of clinical specimens, predicting culture results in 98 per cent of cases for dilutions of urethral samples ranging from 1:400 to 1:1600; the tube method was as accurate for dilutions of only 1:100 and 1:200 of the urethral samples (Prior and Spagna, 1980). At these same dilutions, one (3 per cent) of 39 specimens from patients with NGU gave a positive result with the tube test. With the microdilution method, positive test results were obtained in 33 and 15 per cent of the cases of NGU at dilutions of 1:100 and 1:200 respectively. These higher percentages of positive tests for culture-negative urethral specimens were apparently due to the higher sensitivity of the lysate used in the microdilution test. The minimum sensitivities of the tube and microdilution methods with soluble E. coli endotoxin were the detection of 0.25 and 0.03 ng/ml, respectively. However, it should be noted that in the microdilution test, the incidence of positive results with culture-negative urethral specimens dropped to 3 per cent for dilutions of the specimens ranging

from 1:400 to 1:1600. The Limulus lysate preparations used in both reports (Spagna et al., 1979; Prior and Spagna, 1980) were chloroform extracted by the manufacturer, to remove inhibitors of gel formation for improved sensitivity.

More recently, three Limulus amoebocyte lysate preparations obtained from different suppliers were comparatively evaluated for sensitivity to endotoxin contained in urethral exudates from men with gonococcal urethritis and NGU (Prior and Spagna, 1981). One lysate preparation was not extracted with organic solvents during manufacture, whereas the other two were extracted with chloroform. All three lysate preparations had equivalent sensitivities (0.06 ng/ml) to an established reference endotoxin standard, but significant differences in sensitivities were found among the three preparations when testing clinical specimens. The non-extracted lysate was significantly more sensitive to the presence of endotoxin in gonococcal exudates than the two extracted preparations; it also performed well in the rapid differentiation of gonococcal and non-gonococcal urethritis in men.

Various studies suggest that the diagnosis of some infections is possible by gas-liquid chromatographic analysis of biological specimens (Gorbach et al., 1976; Schlossberg, Brooks and Shulman, 1976; Brooks et al., 1977). In most situations, identification has depended on recognition of specific patterns of metabolites of the organisms or infected tissues. Sud and Feingold (1979) investigated the use of gas-liquid chromatography to establish the presence of organisms in biological specimens by detecting

the presence of unique marker molecules from the organisms rather than patterns of metabolites. These investigators reported a method to identify 3-hydroxy dodecanoic acid in picogram amounts, and applied the method to detect the presence of N. gonorrhoeae. Most Gram-negative bacteria contain 3-hydroxy tetradecanoic acid (or beta-hydroxymyristic acid) which is an integral component of lipid A; neisseriae differ in that 3-hydroxy dodecanoic acid is the major hydroxy acid, and is responsible for 10 to 20 per cent of the total fatty acid content of the cells. The method is limited in that about 10^5 colony-forming-units of N. gonorrhoeae are required for detection. Moreover, interfering substances are a major problem when working at maximum sensitivity of the electron capture detector, thus necessitating complex purification procedures. The method reported is complicated, involving several time consuming procedures.

A gonococcal enzyme, of unknown physiological function, was isolated and characterized (McDonald et al., 1980); it catalyses the conversion of 1,2-propanediol to an unidentified oxidation product in the presence of NAD^+ . The possibility of using this enzyme as a diagnostic marker for N. gonorrhoeae prompted Takeguchi et al. (1980) to survey its distribution among various stock cultures and clinical isolates from the cervix. Results indicated that only members of the genera Neisseria and Acinetobacter possess appreciable activities of the enzyme, and the activity is considerably higher in N. gonorrhoeae. This study suggests that it may be possible to utilize this enzyme as a presumptive diagnostic marker for N. gonorrhoeae in cervical secretions. Further evaluation of this method is required before any conclusions can be drawn.

CONCLUSION

Because of the short incubation period of gonorrhoea and high infectivity, rapid and accurate diagnosis followed by immediate treatment are essential for the control of infection within a community.

Developments in selective media and rapid identification procedures have considerably improved the cultural diagnosis of gonorrhoea (Jephcott and Rashid, 1978; Young, 1978c). When direct plating with immediate incubation is impracticable, several transport and growth systems are now available, in addition to the conventional non-nutrient transport media such as Amies' modification of Stuart's medium.

Unfortunately, all of the above systems depend on maintaining the viability of N. gonorrhoeae, and in many localities transport-associated problems continue to impose limitations on their value. To overcome these problems, it was recommended (World Health Organization, 1978) that methods for the detection of gonococcal components in the secretions of infected patients should be investigated.

Accordingly, this study was undertaken to investigate the value of non-cultural methods in the diagnosis of gonorrhoea. The methods used comprised: genetic transformation, enzyme-linked immunosorbent assay, coagglutination, and detection of endotoxin by the Limulus lysate assay.

MATERIALS AND METHODS

GENERAL

Bacterial strains

N. gonorrhoeae strain F62 colony-type 2 was kindly supplied by Dr A.E. Jephcott, Public Health Laboratory, Myrtle Road, Bristol.

N. gonorrhoeae strain 9 was kindly supplied by Dr D.S. Kellogg, Centre for Disease Control, Atlanta, Ga, USA.

N. gonorrhoeae strain 82409 was kindly supplied by Dr K. Reimann, Neisseria Department, Statens Seruminstitut, Copenhagen.

Stock cultures of the following strains of Neisseria were supplied by the National Collection of Type Cultures, Central Public Health Laboratory, Colindale Avenue, London:

N. elongata NCTC 10660

N. cinerea NCTC 10294

N. catarrhalis NCTC 3622

N. cuniculi NCTC 10297

N. canis NCTC 10296

N. pharyngis var. flavus NCTC 4590

N. pharyngis var. siccus NCTC 4591

N. denitrificans NCTC 10295

N. animalis NCTC 10212

N. meningitidis strains of serogroups A, B, C, D, E, 29E, W135, X and Z were originally obtained from the Neisseria Repository, Berkeley, California.

The following strains were obtained from the laboratory stock cultures of the Department of Bacteriology, University of Edinburgh Medical School:

Streptococcus agalactiae NCTC 11078

Bacteroides bivius VPI 6822

Escherichia coli NCTC 10418

The Oxford staphylococcus, a laboratory stock culture of Pseudomonas aeruginosa, and clinical isolates of N. gonorrhoeae, N. lactamica, and N. perflava were obtained from the service laboratories of the Department of Bacteriology, University of Edinburgh Medical School.

Chemicals, biochemicals, and enzymes

All chemicals and biochemicals used were of pure commercial grade; they were purchased from B.D.H. Chemicals Ltd., Poole, England, or from Sigma London Chemical Company Ltd., Poole, England.

Culture media

MNYC medium	Modified New York City medium was Difco gonococcal base enriched with 10 per cent human blood lysed with 0.5 per cent saponin, 2.5 per cent yeast dialysate prepared as described by Faur <u>et al.</u> (1973b), 0.1 per cent glucose, lincomycin (1.0 µg/ml), colistin (6 µg/ml), amphotericin B (1.0 µg/ml), and
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trimethoprim lactate (6.5 µg/ml).

MNYC medium lacking antibiotics was also used.

GC agar

GC agar was Difco gonococcal base supplemented with L-glutamine, thiamine pyrophosphate, ferric nitrate, and glucose as described by Kellogg et al. (1963), except that the glucose concentration was reduced from 0.4 to 0.2 per cent.

GC broth

The composition of GC broth was the same as GC agar without the agar.

Blood agar

Columbia agar base (Oxoid) containing 5 per cent human blood.

MacConkey agar

MacConkey agar (without salt) (Oxoid).

Modified U-9 medium

The following medium is a modification of that described by Shepard and Luncford (1970):

PFL0 Broth (Difco)	70 ml
Yeast extract	10 ml
Horse serum	20 ml
Urea (50 per cent)	0.2 ml
Lincomycin (1.0 µg/ml)	1.0 ml
Colistin (6 µg/ml)	0.1 ml
Amphotericin B (1.0 µg/ml)	0.1 ml
Trimethoprim lactate (6.5 µg/ml)	0.1 ml
Phenol red (0.2 per cent)	1.0 ml
pH adjusted to 6.5 with 1N HCl.	

Todd Hewitt broth (Oxoid)

Dispensed in bijoux in 2 ml volumes.

Viable counts

Unless otherwise stated, viable counts were performed by making serial ten-fold dilutions of the appropriate suspensions, and spreading 0.1 ml of each dilution over the appropriate growth medium.

Incubation

Standard incubation conditions, unless otherwise stated, were overnight at 37°C in an aerobic CO₂-enriched (10 per cent) and humidified atmosphere.

Confirmatory identification of *N. gonorrhoeae*

Each culture was checked for purity by Gram-staining, and the oxidase reaction was performed by flooding a small area of the surface of the plate with a 1.0 per cent (w/v) solution of tetramethyl-p-phenylenediamine dihydrochloride. This was followed by the fluorescent-antibody test and the rapid carbohydrate-utilization test as described by Young (1978c).

Microscopy

Plates were examined with a Zeiss binocular stereoscopic microscope with substage lighting (Jephcott and Reyn, 1971) in order to determine the colony-type of isolated colonies.

When colony-type 2 gonococci were to be maintained in the

laboratory, daily selective subculturing was performed by selecting single colonies under the microscope and transferring them with a sterile hypodermic syringe needle on to fresh growth medium.

Buffers

Phosphate-buffered saline (PBS) pH 7.2: 10mM phosphate buffer pH 7.2, containing 0.15M NaCl.

PBS pH 7.4: 50mM phosphate buffer pH 7.4, containing 0.15M NaCl.

Isotonic solution

Physiological saline: 0.15M NaCl in distilled water.

SPECIFIC METHODOLOGY

1. TRANSFORMATION STUDIES

Culture media

Gonococcal genetic medium (GGM) (La Scolea and Young, 1974) was used for growth and maintenance of N. gonorrhoeae strain F62 colony-type 2 while it was auxotrophic for proline; the same medium lacking proline (GGM Pro⁻) was used to detect strain F62 when it became prototrophic for proline after transformation.

Preparation of transforming DNA

A sterile swab was charged with the growth from an overnight plate culture of the test organism. It was then placed in 0.5 ml of 0.025 per cent (w/v) sodium dodecyl sulphate in standard saline citrate solution (0.15M sodium chloride, 0.015M sodium citrate) and agitated to remove bacteria. The resulting suspension was heated in a water bath at 65-68°C for 45-60 minutes. After heating, the crude DNA preparations were diluted with 0.3 ml of sterile distilled water to compensate for the possible increase in SDS concentration caused by evaporation.

Transformation assay

Three DNA samples, including DNA extracted from N. gonorrhoeae strain 9 as a control, were tested on a single GGM plate. The plate was divided into seven sections; two drops (approximately 0.04 ml) of a thick suspension (approximately 10^{10} colony-forming units (c.f.u.)/ml) of N. gonorrhoeae strain F62 colony-type 2 in saline citrate were placed on each of three sections of the

plate and one drop on a fourth section. Two drops of each of the three crude DNA preparations were deposited over the recipient cells in each of the three areas that had received two drops of F62. A drop of each of the DNA samples was placed on the remaining three sections as a sterility control. After incubation of the plate for 3 hours at 37°C in a CO₂-enriched (10 per cent) humidified atmosphere, the contents of each section were streaked on to an identically marked plate of GGM Pro⁻ medium which was then incubated for 48 hours under standard conditions. The GGM plate was also incubated to check the viability of F62 and to monitor for contamination.

After incubation, the GGM Pro⁻ plates were examined with a Zeiss binocular stereoscopic microscope with substage lighting (Jephcott and Reyn, 1971). A positive result in the transformation assay was indicated by growth of several colonies (≥ 20 colonies) identical in size and appearance to the colonies of strain F62 growing on GGM. The results of the test specimens were compared with those obtained with N. gonorrhoeae strain 9. The section containing non-DNA-treated strain F62 streaked on to GGM Pro⁻ was a control to detect spontaneous revertant prototrophic cells.

Relationship of colony-type of recipient to the efficiency of transformation

Viable counts were made on thick suspensions of N. gonorrhoeae strain F62 colony-type 2 and 3 by the method of Miles, Misra and Irwin (1938); the dilutions were made in saline citrate and the

colonies counted on clear GC agar. A crude DNA preparation was made from N. gonorrhoeae strain 9. Hundred-fold dilutions of the DNA preparation were made in saline citrate up to a dilution of 10^{12} and assayed as already described with cells of colony-types 2 and 3 as recipients.

Assessment of sensitivity of the test procedure with N. gonorrhoeae strain 9 and N. meningitidis serogroup B

A crude DNA preparation was made from a thick suspension of N. gonorrhoeae strain 9; the number of bacteria present in the suspension was determined by the method of Miles et al. (1938). Hundred-fold dilutions of the crude DNA were made up to a dilution of 10^{12} . Each dilution was assayed as previously described. The procedure was repeated with N. meningitidis serogroup B as test organism.

Specificity of the test procedure

Crude DNA preparations were made from thick suspensions of all of the laboratory stock cultures of non-gonococcal neisseriae listed earlier, the Oxford staphylococcus, and Ps. aeruginosa.

This procedure was repeated with subcultures of genital and oral isolates of neisseriae from patients attending the Department of Genito-Urinary Medicine, the Royal Infirmary, Edinburgh; these included 169 strains of N. gonorrhoeae, 12 of N. meningitidis, three of N. lactamica, and one of N. perflava.

Assessment of proportion of clinical isolates auxotrophic for proline

Eighty-four of the clinical isolates were subcultured on to GGM and GGM Pro⁻ media to test their requirement for proline.

Duration of storage of test organisms on swabs

Nineteen MNYC plates were seeded with N. gonorrhoeae strain 9. After overnight incubation, the growth on each plate was harvested with a sterile serum-coated swab (Exogen Ltd., Clydebank Industrial Estate, Beardmore Street, Clydebank G81 4SA, Scotland). Nine of the swabs were stored at 4°C, nine at room temperature, and the remaining swab was used immediately in a transformation assay. The test organisms on the swabs, stored as described above, were assayed at intervals up to 68 days.

Transformation assay with swab specimens from men and women

Urethral swab specimens were obtained from 42 men attending the Department of Genito-Urinary Medicine, the Royal Infirmary, Edinburgh.

Cervical swab specimens were obtained from 23 contacts of men with gonorrhoea, attending the Department of Genito-Urinary Medicine, the Royal Infirmary, Edinburgh.

2. DETECTION OF GONOCOCCAL ANTIGENS BY THE ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

2.1 Preparation of antisera against whole untreated cells

Antisera against whole, untreated N. gonorrhoeae strains 9 and 82409 (both colony-type 2) were raised in New Zealand white rabbits. The bacteria were grown overnight on GC agar, harvested, and suspended in PBS pH 7.2. The two suspensions were aseptically adjusted to an extinction value of 1.0 at 450nm (equivalent to 10^9 c.f.u./ml), and stored in 0.5 and 1.0 ml aliquots at -20°C until required; one aliquot was thawed per injection.

Prior to the first injection, a test-bleed was taken to check the base line antibody level. The rabbits received intravenous injections according to the following schedule: week 1, daily 0.5 ml injections for 4 days; week 2, daily 1.0 ml injections for 5 days; week 3, no injections; week 4, daily 1.0 ml injections for 5 days; week 5, test bleed (5 ml) then 2 days later the rabbits were exsanguinated by cardiac puncture.

Antisera against whole, untreated N. gonorrhoeae strain 9 colony-type 2 were raised in CFI (albino) randomly bred mice. The mice received 0.2 ml intravenous injections containing 10^9 c.f.u./ml, prepared as described previously, according to the following schedule: week 1, daily injections for 3 days; week 2, daily injections for 3 days; week 3, no injections; week 4, daily injections for 3 days; week 5, daily injections for 3 days;

week 6, test bleed (2 ml) then 2 days later the mice were exsanguinated.

The antibody titres were determined by the gonococcal complement fixation test (GCFT) as described by Young, Henrichsen, and McMillan (1980).

2.2 Preparation of antigens

Growth of bacteria

An overnight culture of the appropriate strain of N. gonorrhoeae maintained in the laboratory by daily subculture was used to inoculate one hundred plates of GC medium. Inoculation was carried out by charging a sterile swab with several colonies of bacteria, and using this to inoculate a series of ten plates: nine plates were inoculated to provide confluent growth, while the tenth plate was inoculated to provide single colonies. This procedure was repeated to give 100 plates. After inoculation, plates were incubated overnight under standard conditions.

Harvesting

Prior to harvesting the overnight growth, all the plates were checked macroscopically for possible contamination; the identity of the organisms on the tenth plate in each series was confirmed by the standard methods.

The cell paste was brought to the centre of each plate by scraping the agar surface with a microscope slide: the slide

was dipped in to absolute alcohol and flamed at regular intervals. To avoid drying, once twenty plates were treated in this way, the cell paste was transferred with a sterile loop in to ice cold PBS pH 7.4.

Lipopolysaccharide (LPS) extraction and purification

LPS was extracted from N. gonorrhoeae strain 9 colony-type 3 by the aqueous phenol procedure of Perry et al. (1975). The cells were harvested and washed twice in PBS pH 7.4. The second wash was performed in a pre-weighed tube in order to get an estimate of the cell yield.

The cell paste was resuspended in 200 ml ice cold distilled water, and the mixture was dispersed by blending in a top-drive Omnimixer operated at top speed for 10 seconds. An equal volume of 90 per cent (w/v) phenol in water was added to the suspension. The mixture was again dispersed by blending three times for a total of 30 seconds. It was then incubated at 65°C and stirred vigorously for 10 minutes. After cooling in ice, the mixture was centrifuged at 1000g for 30 minutes at 4°C.

The upper aqueous phase which contained free LPS, glycogen, and nucleic acids was carefully separated with a pasteur pipette and dialysed for 24 hours against running tap water to remove the phenol. The LPS was removed from solution by rotary evaporation to reduce the volume, followed by centrifugation at 100 000g for 3 hours at 4°C. The LPS was purified by resuspending in a small volume of distilled water, followed

by centrifugation at 125 000g for 4 hours at 4°C. The pellet was resuspended in 5 ml distilled water and freeze-dried.

Preparation of whole cell (GCFT) antigen

The GCFT antigen was prepared from N. gonorrhoeae strain 9 colony-type 3 as described by Young et al. (1980).

The overnight growth from ten plates of GC medium was harvested in to 10 ml 0.9 per cent sterile physiological saline, washed once by centrifugation, and resuspended in 10 ml 0.9 per cent physiological saline. The purity of the suspension was checked by Gram and immunofluorescence staining, and by culture. The suspension was then heated in a water bath at 60°C for 30 minutes with mixing every 5 minutes. After cooling to room temperature, the suspension was stored at 4°C.

Preparation of outer membrane (OM) complex

OM complex preparations were made from the following organisms:

N. gonorrhoeae strain 9 (colony-type 3)

N. gonorrhoeae strain 82409 (colony-type 2)

Ten clinical isolates of N. gonorrhoeae

N. meningitidis serogroup B

N. lactamica

N. perflava

N. pharyngis var. flavus

Streptococcus agalactiae

Bacteroides bivius

Escherichia coli

The procedure was the same as that described by Poxton (1979). The bacteria were washed twice in PBS pH 7.4 by centrifugation for 30 minutes at 4°C at 10 000g using a Sorvall RC-5B centrifuge fitted with an SS-34 centrifuge head. The pellets were resuspended in 50 ml PBS pH 7.4 containing 10mM EDTA, incubated at 45°C for 30 minutes, and treated in an ultrasonic bath (Model 6441A, Dawe Instruments Ltd., Western Avenue, London W3 0SD, UK) for 60 seconds. The treated cells were removed by centrifugation at 10 000g for 30 minutes, leaving the OM complex in solution. Dialysis was not required since the antigen was to be diluted for use in the enzyme-linked immunosorbent assay.

Quantitative analytical procedures

Protein concentration was determined by the method of Lowry et al. (1951), with bovine serum albumin as standard.

Total carbohydrate was determined by the phenol/sulphuric acid procedure of Dubois et al. (1956), with glucose as standard.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The procedure was essentially the same as that of Poxton and Brown (1979). Slab gels (170 x 140mm) of 10 per cent (w/v) acrylamide with a 10mm 4 per cent (w/v) stacking gel were used to run up to 12 samples in a Raven (Haverhill, Suffolk, CB9 7UU, UK) slab gel apparatus. The buffers used were as described by Laemmli (1970). Protein samples at a concentration of 1mg/ml were solubilized by heating in a boiling water bath for 3 minutes

in a SDS-PAGE sample buffer, containing 2 per cent (w/v) SDS (Poxton and Brown, 1979) and bromophenol blue, just prior to the application to the gel. Samples containing 50 µg protein were applied to each well. Standards (Molecular weight markers for SDS-PAGE, BDH Chemicals Ltd., Poole BH12 4NN, England) were included in each gel. Electrophoresis was carried out at a constant voltage of 50 V until the samples entered the separating gel (approximately 1 hour), and was then maintained at 150 V until the bromophenol blue was near the bottom of the gel (approximately 3 hours). The gels were stained for protein with Coomassie Brilliant Blue (Poxton and Sutherland, 1976).

2.3 Enzyme-linked immunosorbent assay (ELISA)

ELISA procedure

This procedure was essentially the same as that described by Engvall and Perlmann (1972) but adapted for microtitre plates (flat-well plates supplied by Sterilin Ltd., Teddington, Middlesex, UK) as described by Voller et al. (1976) and Poxton (1979).

Fifty microlitres of antigen in 50mM carbonate buffer pH 9.6, containing 0.02 per cent sodium azide were added to each well and incubated at 37°C for 4 hours, then held at 4°C overnight. The plate was washed three times with 0.15M NaCl containing 0.05 per cent Tween 20, by directing the nozzle of a wash-bottle in to each well and filling it to the top. The plates were shaken dry between each wash. Antiserum, diluted in PBS pH 7.4

containing 0.05 per cent Tween 20 and 0.02 per cent sodium azide, was added to each well (50 μ l) and incubated at 37°C for 1 hour. The plates were washed as before and anti-rabbit IgG conjugated to alkaline phosphatase (Miles Laboratories (UK) Ltd., Stoke Poges, Slough, Bucks, UK), diluted in the same buffer as the antiserum, was added to each well (50 μ l) and incubated at 37°C for 1 hour. After washing the plates, 50 μ l volumes of the enzyme substrate (1 mg/ml solution of p-nitrophenylphosphate (Sigma) in 50mM carbonate buffer pH 9.8, containing 1mM MgCl_2) were added to each well. After incubation at 37°C for 1 hour, the extinction values were read at 405nm using a Titertek^R Multiskan (Organon Teknika, Teknika House, Cromwell Road, St. Neots, Huntingdon, Cambridgeshire PE19 2EU).

Initially, the following negative controls were set up on every plate:

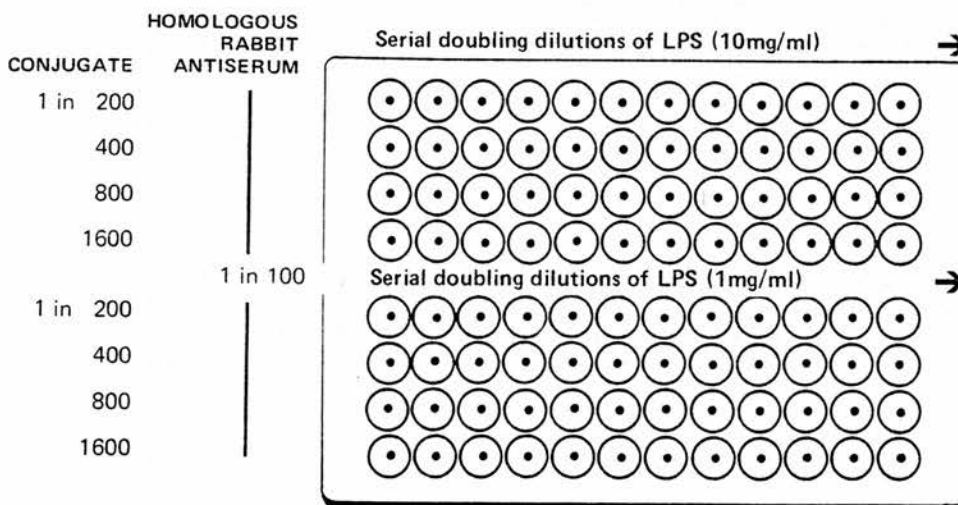
Controls	Antigen	Rabbit antiserum	Conjugate	Substrate
1	+	-	+	-
2	+	-	-	+
3	-	+	+	+
4	-	+	+	-
5	-	-	+	+
6	-	-	-	+

Microtitre plates coated with LPS

Freeze-dried purified LPS from N. gonorrhoeae strain 9 was reconstituted in distilled water to a concentration of 10 mg/ml. An aliquot of this was diluted ten-fold to a concentration of 1 mg/ml.

To determine the amount of LPS required to coat the wells, and a suitable dilution for the anti-rabbit IgG-alkaline phosphatase, half a microtitre plate was coated with doubling dilutions of LPS at the starting concentration of 10 mg/ml (500 µg/50 µl); the other half was coated with doubling dilutions of LPS at the starting concentration of 1 mg/ml (50 µg/50 µl). The rabbit serum was used at a dilution of 1 in 100, and the conjugate at dilutions of 1 in 200, 400, 800, and 1600.

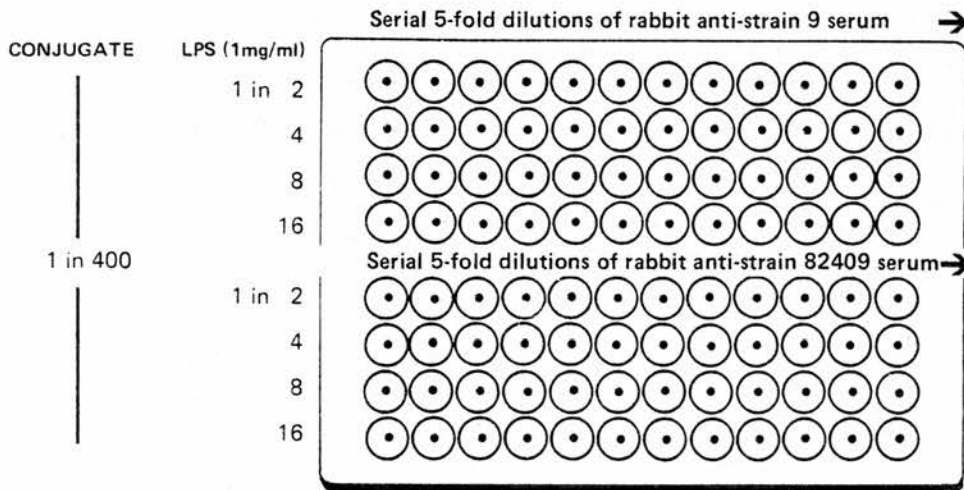
The following (Fig. 2) is a diagrammatic representation of the experiment:



Determination of antibody titres of rabbit antisera with purified gonococcal LPS as antigen

The assay was carried out to determine the antibody titres of the rabbit antisera raised against N. gonorrhoeae strains 9 and 82409 when LPS from strain 9 was used as antigen.

The following (Fig. 3) is a diagrammatic representation of the experiment:



Determination of antibody titres of rabbit antisera in ELISA tests with whole cell (GCFT) antigen

Five-fold dilutions of the GCFT antigen prepared from N. gonorrhoeae strain 9 and ranging from 1 in 10 to 1 in 1250 were made.

Rabbit sera were diluted five-fold, and conjugate was used at the fixed dilution of 1 in 400.

Effect of OM complex protein on extinction

Serial doubling dilutions of the OM complex preparation from N. gonorrhoeae strain 9 (protein concentration of 1 mg/ml) were used to coat the wells over a range of dilutions from 1 in 2 to 1 in 32 768.

Homologous rabbit serum and conjugate were used at dilutions of 1 in 5000 and 1 in 400 respectively.

OM complex preparations from N. gonorrhoeae strains 9 and 82409 tested against homologous and heterologous rabbit antisera

OM complex preparations (protein concentration of 1 mg/ml) from N. gonorrhoeae strain 9 and 82409 were diluted ten-fold. Each well received 50 µl of antigen, equivalent to approximately 5 µg protein.

Each of two sets of wells was coated with one of the OM complex preparations. The antibody titres of both rabbit antisera were determined against homologous and heterologous antigens, in order to determine the degrees of cross-reactivity.

ELISA-inhibition with purified LPS

The wells of a microtitre plate were coated with N. gonorrhoeae strain 9 OM complex (protein concentration of 5 µg/50 µl). Conjugate was used at a dilution of 1 in 400.

Undiluted and a ten-fold dilution of antiserum raised against N. gonorrhoeae strain 9 were mixed with an equal volume of LPS (1 mg/ml). The bijoux containing the mixtures were fitted on to a rotor with a vertical axis operated at 27 rpm, and incubated

at 37°C for 4 hours; incubation was continued overnight at 4°C.

The controls consisted of untreated rabbit serum titrated on the same microtitre plate; rabbit serum preincubated with PBS pH 7.4 instead of antigen.

Antibody titres of rabbit antiserum obtained with various OM complex preparations as antigen

Each of the OM complex preparations (protein concentration of 1 mg/ml) from the following organisms was used to coat a separate row of wells:

N. gonorrhoeae strain 9

10 clinical isolates of N. gonorrhoeae

N. meningitidis serogroup B

N. lactamica, N. perflava, and N. pharyngis var. flavus

S. agalactiae, B. bivius, and E. coli

The antibody titre of the rabbit anti-strain 9 serum was determined against each of these antigens. Doubling dilutions of the rabbit antiserum starting at 1 in 50, and conjugate at a dilution of 1 in 400 were used.

Determination of specificity by ELISA-inhibition

A ten-fold dilution of the rabbit anti-strain 9 serum was aliquoted in to separate bijoux. An equal volume of each of the OM complex preparations (protein concentration of 1 mg/ml) used in the preceding experiment was mixed separately with the rabbit antiserum. The bijoux containing the mixtures were

fitted on to a rotor with vertical axis operated at 27 rpm, and incubated at 37°C for 4 hours; incubation was continued overnight at 4°C.

The wells were coated with N. gonorrhoeae strain 9 OM complex (protein concentration of 1 mg/ml). Conjugate was used at a dilution of 1 in 400.

The controls consisted of untreated rabbit serum titrated on the same microtitre plates; rabbit serum preincubated with FBS pH 7.4 instead of antigen.

2.4 Indirect sandwich ELISA for the detection of N. gonorrhoeae

This procedure was essentially the same as that described by Drow, Maki, and Manning (1979).

Polystyrene balls (0.25 in., ca 0.6 cm) with frosted finish, RIA grade (Euro-Matic Ltd., Maycrete House, Boston Manor Road, Brentford, Middlesex, England) were armed with mouse antiserum, raised against whole untreated cells of N. gonorrhoeae strain 9, by immersing them in the antiserum diluted in 50mM carbonate buffer pH 9.6, containing 0.02 per cent sodium azide. Incubation was carried out at 37°C for 4 hours in a shaking incubator, and at 4°C overnight. Supernatant fluid was removed by suction with a Pasteur pipette attached to a vacuum source, and the balls were washed three times with 0.15M NaCl containing 0.05 per cent Tween 20.

The washed balls were then immersed in 10 per cent fetal

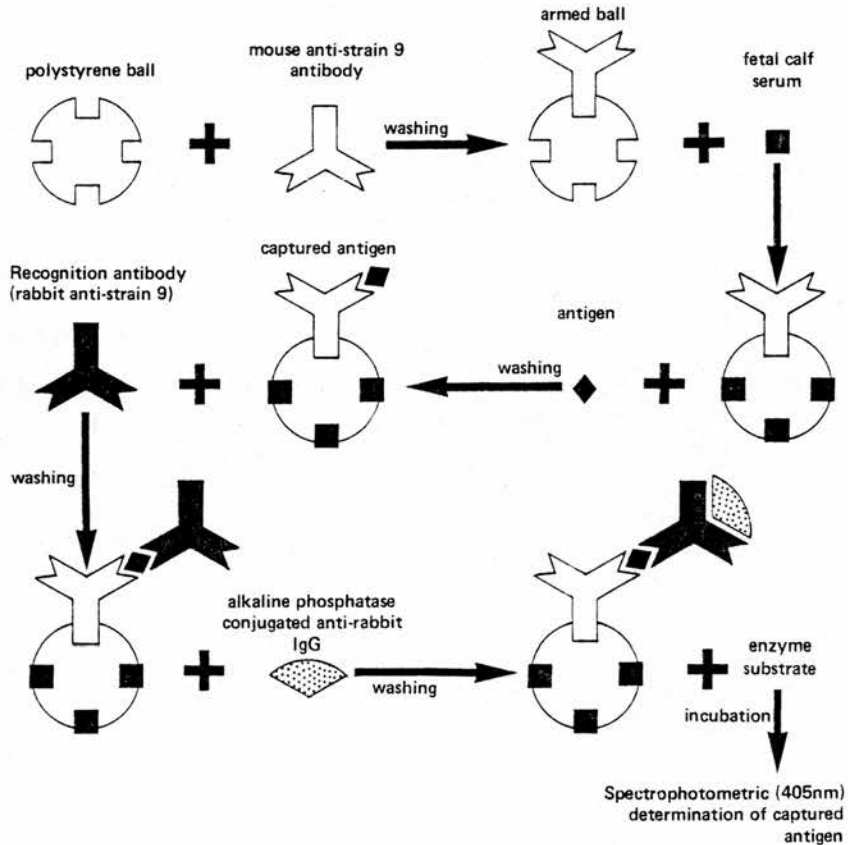
calf serum (FCS) in PBS pH 7.4 with 0.2 per cent sodium azide; incubation was carried out at room temperature for 1 hour. The FCS was then removed by suction, and the balls placed on gauze in petri dishes for rapid drying. The armed balls were stored at 4°C.

On the day of the assay, armed balls were placed in glass tubes (72"x 12mm) (one ball/tube). For antigen capture, 300 μ l of antigen in PBS pH 7.4, containing 0.05 per cent Tween 20 and 0.02 per cent sodium azide, was added to each tube. After incubation in a 40°C water bath for 1 hour, the balls were washed three times. Captured antigen was recognized by the addition of 300 μ l rabbit anti-strain 9 serum diluted in PBS pH 7.4, containing 0.05 per cent Tween 20 and 0.02 per cent sodium azide. Incubation was carried out at 40°C for 1 hour. After washing as before, 300 μ l of anti-rabbit IgG conjugated to alkaline phosphatase, diluted in the same buffer as that of the rabbit antiserum, was added to each tube. After incubation at 40°C for 1 hour, the balls were washed as before and transferred to clean glass tubes. 300 μ l volumes of the enzyme substrate (1 mg/ml solution of p-nitrophenylphosphate in 50mM carbonate buffer pH 9.8, containing 1mM MgCl_2) were added to each tube. After incubation at 40°C for 1 hour, the solutions were quickly transferred to a microtitre plate, and the extinction values read at 405nm using a Titertek^R Multiskan.

Negative controls were set up in duplicate with every

experiment; they comprised antigen diluent instead of antigen and were otherwise treated in the usual manner.

The assay can be represented schematically as follows:



Dilutions of mouse and rabbit antisera to be used

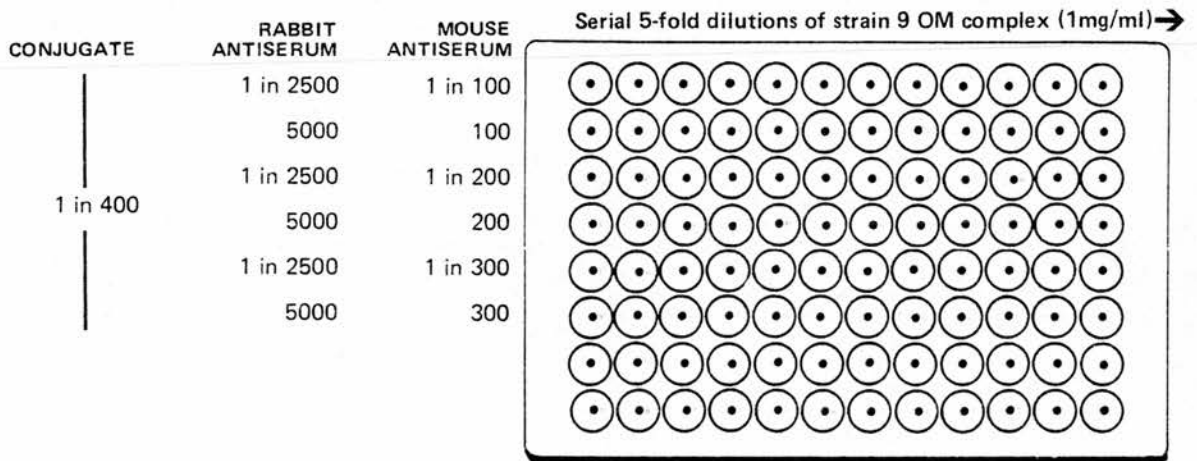
Three sets of polystyrene balls (24/set) were armed with mouse anti-strain 9 serum at dilutions of 1 in 100, 200, and 300.

Rabbit anti-strain 9 serum at dilutions of 1 in 2500 and 5000, and conjugate at a dilution of 1 in 400 were used in the assay.

N. gonorrhoeae strain 9 OM complex (protein concentration of 1 mg/ml) was serially diluted five-fold in order to determine

the minimum amount of protein detectable with the various dilutions of the mouse and rabbit antisera.

The following (Fig. 6) is a diagrammatic representation of the experiment:



Specificity and sensitivity of the assay using OM complex preparations

The polystyrene balls were armed with mouse anti-strain 9 serum at a dilution of 1 in 300.

Rabbit antiserum and conjugate were used at dilutions of 1 in 2500 and 1 in 400 respectively.

The specificity of the assay and the minimum amount of OM complex protein detectable with this system were determined using OM complex preparations (protein concentration of 1 mg/ml) from N. gonorrhoeae strain 9, 10 clinical isolates of N. gonorrhoeae, N. meningitidis serogroup B, S. agalactiae, B. bivius, and E. coli.

The antigens were diluted ten-fold, and the titrations were carried out by serially diluting them two-fold.

Negative controls consisted of armed balls treated in the same manner except for the use of antigen diluent instead of antigen.

Detection of whole cell antigen as a preliminary to investigating clinical specimens

N. gonorrhoeae strain 9 from an overnight growth on GC medium was suspended in 1ml physiological saline. A 100 μ l volume of the suspension was immediately used to perform a viable count on GC medium.

After holding the suspension at room temperature for 2 hours, 100 μ l of 500mM phosphate buffer pH 7.4 containing 0.85 per cent NaCl, 0.5 per cent Tween 20, and 0.2 per cent sodium azide, was added to the 0.9 ml suspension; the 10x concentrated buffer (antigen diluent) was therefore diluted ten-fold, bringing it to its usual concentration.

To the 1ml suspension was added an equal volume of Sputolysin (Calbiochem-Behring Corp., La Jolla, Ca 92037), and the mixture was serially diluted ten-fold; 300 μ l of each dilution was added to an appropriately labelled tube containing one armed ball, and the assay was carried out as described.

Negative controls consisted of balls treated in the same manner except for the use of antigen diluent instead of antigen.

3. DETECTION OF GONOCOCCAL COMPONENTS WITH COMMERCIALY AVAILABLE REAGENTS

3.1 Detection of endotoxin in clinical material by the Limulus lysate assay

Study population

Sixty six women attending the Department of Genito-Urinary Medicine, Black Street Clinic, Glasgow, were investigated. Specimens were taken from the urethra and cervix for microscopic examination and culture, and from the rectum for culture only. Identification methods for N. gonorrhoeae were as already described.

Collection of secretions for the Limulus lysate assay

Under direct vision, the cervix was cleaned with a cotton-wool swab held in sponge-holding forceps. Secretions were collected from the endocervical canal by gentle aspiration through a sterile pyrogen-free polythene capillary tube (chromatography column tubing, internal diameter 1.0mm, obtained from Pharmacia Fine Chemicals, Uppsala, Sweden), one end of which had been inserted through the os to about 1 cm; the other end of the tube was attached to a 5 ml syringe containing 1 ml pyrogen-free water which was ejected in to the endocervical canal. After allowing for equilibration with endocervical secretions for 30 seconds, the combined washings were aspirated in to the same syringe and ejected in to a pyrogen-free plastic container. The secretions were stored at -20°C until required.

Limulus assay

Frozen specimens of cervical secretions were thawed and mixed thoroughly with a vortex mixer operated at full speed; they were then diluted in pyrogen-free water to a final dilution of 1 in 100.

0.1 ml of the diluted secretion was mixed in a pyrogen-free plastic container with 0.1 ml of reagent reconstituted from the 50 Test Pyrotest TM vial (Difco Laboratories, Detroit, Michigan); the mixture was incubated at 37°C in a water bath for one hour. Results were interpreted in accordance with the manufacturer's instructions: a firm opaque gel which remained adherent to the bottom of the vial when inverted through 180° was scored positive; the absence of a firm gel was scored negative. Known positive and negative control samples were also tested with each batch of assays. The sensitivity of the Limulus assay was demonstrated by the detection of 0.125 ng/ml Difco Pyrotrol positive control E. coli endotoxin.

The Limulus assays were read without previous knowledge of the conventional microbiological results. After the correlation between the Limulus assay results and a diagnosis of gonorrhoea had been made, certain specimens showing an apparently false-positive Limulus result were re-tested at a dilution of 1 in 200, while specimens showing a false-negative result were re-tested at a dilution of 1 in 50.

*1.5 x 4.0 cm

Protein estimation

In order to determine whether false-negative results could possibly be due to the secretions being very dilute, the protein concentration of several secretions (four false-negative and five confirmed positive specimens chosen at random) was determined by the method of Lowry et al. (1951).

Statistical analysis

The Chi Square Test with Yates's correction was used to test the correlation between Limulus assay results and conventional microbiological findings.

The Student's t test was used to compare the mean protein concentrations in secretions giving false-negative and confirmed positive Limulus results.

3.2 Coagglutination test

Coagglutination reagents were prepared by Pharmacia Diagnostics AB, Uppsala, Sweden, by the method of Danielsson and Kronvall (1974). The test reagent, which consisted of rabbit anti-gonococcal serum bound to dead staphylococci, was prepared with the same batch of antiserum raised against N. gonorrhoeae strain 9 as used for ELISA. It was not absorbed to remove cross-reaction with N. meningitidis, and was supplied in liquid form containing methylene blue. The control reagent consisted of gamma globulin, from non-immunized rabbits, bound to dead staphylococci.

Sensitivity of coagglutination test

The sensitivity of the assay was tested using N. gonorrhoeae strain 9 and N. meningitidis serogroup B.

The same procedure was carried out with each of the two organisms. A heavy suspension of an overnight culture was made by removing the growth from the culture plate with a sterile cotton-tipped swab and emulsifying it in 1 ml sterile distilled water in a tube. One hundred microlitres of the suspension was serially diluted ten-fold in sterile physiological saline, and a viable count performed on MNYC medium.

The tube containing the undiluted suspension was covered and the suspension heated in a boiling water bath for five minutes. The suspension was then serially diluted two-fold in distilled water up to a dilution of 10^{10} . The coagglutination test was then performed by mixing 40 μ l aliquots of suspension with an equal volume of test reagent, and control reagent, on a clear glass slide; three tests were carried out on a single slide (10.7 cm x 5.0 cm) with six clearly defined areas. The slide was rocked gently for two to three minutes by tilting it to an angle of 45° every two to three seconds. Slides were read against a dark background using indirect light.

A significantly stronger reaction in the gonococcal reagent compared with the control reagent constituted a positive result. No reaction in the test reagent irrespective of any reaction in the control reagent constituted a negative result. A reaction of equal strength in both the gonococcal and the control reagents constituted a non-interpretable result.

4. MICROFLORA OF CERVIX AND VAGINA OF PATIENTS WITH AND WITHOUT GONORRHOEA

4.1 Study population

Thirty seven women attending the Department of Genito-Urinary Medicine, the Royal Infirmary, Edinburgh, were investigated. Specimens were taken from the urethra and cervix for microscopic examination and culture, and from the rectum for culture only. Cultures were inoculated on to MNYC medium at the time of the patient examination and the plates transported to the laboratory within three hours. Culture and identification methods for N. gonorrhoeae were as described by Young (1978a).

Wet films of vaginal secretions were examined microscopically for the detection of Trichomonas vaginalis, and Gram-stained smears for Candida albicans.

The isolation of Chlamydia trachomatis was by the method of Thomas et al. (1977) with cycloheximide-treated cells. Incubation of the infected cells was continued for three days at 35°C, and the coverslips stained with iodine to detect the glycogen inclusions.

4.2 Collection of cervical and vaginal secretions

Cervical secretions from patient 1 to 15 were collected as previously described for the Limulus lysate assay. From patient 16 onward, the secretions were aspirated with pyrogen-free physiological saline in place of distilled water.

Under direct vision, the vagina was cleaned with a cotton-wool swab held in a sponge-holding forceps. Secretions were collected from the vagina by gentle aspiration through a sterile polythene capillary tube (chromatography column tubing, internal diameter 1.0mm, obtained from Pharmacia Fine Chemicals, Uppsala, Sweden), one end of which had been inserted in to the posterior fornix of the vagina; the other end of the tube was attached to a 5 ml syringe containing 5 ml pyrogen-free physiological saline which was ejected in to the vagina. After allowing for equilibration with vaginal secretions for 30 seconds, the combined washings were aspirated in to the same syringe and ejected in to a sterile plastic Universal container.

4.3 Bacteriological investigation of cervical and vaginal specimens

Viable counts

Immediately after collection, cervical and vaginal secretions were mixed thoroughly with a vortex mixer operated at full speed.

Secretions were diluted in GC broth up to a dilution of 10^5 , and viable counts made by spreading 0.1 ml of each dilution on the following media: modified New York City medium (MNYC), MacConkey (MacC), and blood agar (BA). The counts on the latter medium were performed in duplicate and incubated anaerobically as well as aerobically. Plates were examined after 24 and 48 hours incubation.

For the isolation and identification of Ureaplasma urealyticum,

100 µl of each specimen was aseptically transferred to 0.9 ml of modified-U9 medium. Serial ten-fold dilutions were made up to a dilution of 10^{10} . Incubation was carried out under standard conditions, and the vials were examined at 24 and 48 hours. The presence of U. urealyticum was indicated by a colour change in the medium from yellow to pink. One colour-changing-unit (c.c.u.) of activity was defined as the highest dilution of the ureaplasma suspension that produced a colour change (Taylor-Robinson *et al.*, 1971).

Aliquots (200 µl) of cervical and vaginal secretions were dispensed in small plastic containers; enough material was left in the pyrogen-free vials for the Limulus lysate assay. The secretions were stored at -20°C until required.

Criteria for identification of isolates from cervix and vagina

N. gonorrhoeae

Growth on MNYC medium.

Standard identification methods were carried out as described.

The following organisms were identified according to the criteria outlined in Cowan and Steel's Manual for the identification of medical bacteria (Cowan, 1977).

Staphylococcus

Growth on BA and MacC.

Facultative anaerobiosis.

Large white or golden yellow, sharply defined convex colonies.

Gram-positive cocci in irregular clusters.

Coagulase test for the identification of S. aureus.

Haemolytic streptococci

Growth on BA.

Facultative anaerobiosis.

Large white mucoid, sharply defined convex colonies, surrounded by a wide clear zone of haemolysis (beta-haemolysis).

Penicillin sensitivity (using 1 unit disks).

Small Gram-positive cocci, characteristically growing in chains.

Subculture in to Todd-Hewitt broth (inoculum from broth on to BA to check for purity of suspension).

Coagglutination test (Phadebact Streptococcus Test, Pharmacia (Great Britain) Ltd.) with samples from Todd-Hewitt broth for identification of Lancefield's groups A, B, C, D, and G.

Enterococci (Streptococcus faecalis)

Growth on BA and MacC.

Characteristic magenta colonies on MacC.

Facultative anaerobiosis.

Penicillin resistance (using 1 unit disks).

Lancefield's group D by coagglutination (Phadebact Streptococcus Test).

Lactobacilli

Growth on BA.

Facultative anaerobiosis.

Very small opaque colonies with slight haemolysis.

Small fat Gram-positive rods with rounded ends.

Catalase negative.

Diphtheroids

Growth on BA.

Facultative anaerobiosis.

Small white colonies with variable haemolysis.

Gram-positive non-motile bacilli, slender and granulated, with Chinese letter configuration.

Catalase positive.

Variable urease production.

Glucose, maltose, lactose, mannite, dulcitol, and sucrose fermentation.

Most Gram-positive bacilli which were catalase positive and urease positive did not utilize any of the sugars tested, and were considered to be Corynebacterium hofmannii.

A few isolates with typical diphtheroid-like colonies stained Gram-negative, were catalase negative and haemolytic; these were presumptively classified as Corynebacterium vaginale.

Proteus

Thin spreading growth on the surface of BA.

Growth on MacC: non-lactose fermenter.

Facultative anaerobiosis.

Gram-negative motile bacilli.

Urease production.

Escherichia coli

Growth on BA.

Growth on MacC: lactose fermenter.

Facultative anaerobiosis.

Gram-negative motile bacilli.

Sugar fermentation: inositol negative.

Anaerobic streptococci

Growth on BA.

Obligate anaerobiosis.

Small Gram-positive cocci.

Bacteroides spp.

Growth on BA.

Obligate anaerobiosis (metronidazole-sensitive).

Small Gram-negative bacilli.

Identification by sugar fermentation and other biochemical tests as described by Duerden et al. (1980).

Clostridium

Growth on BA.

Obligate anaerobiosis.

Large fat Gram-positive bacilli with granulated appearance.

Very large zones of haemolysis (double-zone effect).

Penicillin sensitive (using 1 unit disks).

Candida albicans

Growth on BA.

Facultative anaerobiosis.

Gram-positive, large, oval or spherical cells.

Growth on Sabouraud's agar.

Germ tube positive: a suspension was made in human serum, incubated at 37°C in a water bath, and examined every hour for the presence of germ tubes (hyphae) by making a wet film.

5. DETECTION OF GONOCOCCAL COMPONENTS IN THE CERVICAL AND VAGINAL SECRETIONS INVESTIGATED BACTERIOLOGICALLY

One aliquot from each of the 37 cervical and vaginal aspirates was thawed. All the specimens were processed in one batch by each of the four assays described.

5.1 Transformation assay

To each 200 μ l of aspirate, 200 μ l of saline citrate (pH 7.4) containing 0.05 per cent SDS was added and mixed thoroughly with a vortex mixer operated at full speed. The mixtures were incubated at 65°C for 1 hour and processed as described (p.97) using N. gonorrhoeae strain F62 as recipient.

5.2 Indirect sandwich ELISA

To 180 μ l of aspirate, 20 μ l of PBS (pH 7.4) was added; the molarity of the phosphate buffer was increased from 0.05M to 0.5M to allow for the ten-fold dilution in aspirate. NaCl was kept at 0.15M since the secretions were aspirated with physiological saline. The concentrations of Tween 20 and sodium azide were increased to 0.5 per cent and 0.2 per cent respectively. After the addition of 200 μ l of sputolysin, the specimens were mixed thoroughly by vortexing and processed as described (p.113). Positive and negative controls were set up in duplicate; negative controls comprised antigen diluent instead of antigen, positive controls comprised a suspension of N. gonorrhoeae strain 9 in

saline which was treated in the same manner as clinical specimens.

5.3 Limulus lysate assay

The assay was performed on the cervical secretions collected in pyrogen-free containers. The specimens were mixed thoroughly by vortexing, diluted in pyrogen-free water to a final dilution of 1 in 100, and processed as described (p.119).

After the correlation between the Limulus assay results and a diagnosis of gonorrhoea had been made, certain specimens showing an apparently false-positive Limulus result were re-tested at a dilution of 1 in 200, while specimens showing a false-negative result were re-tested at a dilution of 1 in 50.

Finally all the specimens showing a true-positive result at a dilution of 1 in 100 were re-tested at a dilution of 1 in 200.

5.4 Coagglutination test

Cervical specimens were thawed and boiled for five minutes. After cooling to room temperature, the test was carried out with 40 μ l aliquots as described (p.121).

RESULTS

1. TRANSFORMATION STUDIES

Colony-type of recipient in relation to the efficiency of transformation

Viable counts showed that suspensions of N. gonorrhoeae strain F62 colony-types 2 and 3 used as recipient contained approximately 10^{10} c.f.u./ml. With a series of hundred-fold dilutions of a crude DNA preparation made from N. gonorrhoeae strain 9, up to a dilution of 10^{12} , strain F62 colony-type 2 gave a positive transformation result up to a dilution of 10^6 DNA whereas colony-type 3 gave a positive transformation result up to a dilution of 10^4 .

Stock strains

Apart from N. gonorrhoeae strain 9 and N. meningitidis strains of serogroups A, B, C, D, E, 29E, W135, X, and Z, all of the remaining stock cultures, including the various other members of the family Neisseriaceae failed to transform N. gonorrhoeae strain F62.

Clinical isolates

Of the 169 clinical isolates of N. gonorrhoeae, 150 (88.8 per cent) gave a positive transformation assay. All 12 of the meningococcal isolates tested were positive as were the three strains of N. lactamica. N. perflava gave a negative transformation result.

Assessment of proportion of clinical isolates auxotrophic for proline

Of 84 clinical isolates of gonococci tested, 73 gave a positive transformation assay. Eighteen of the isolates grew on GGM and on GGM Pro⁻, 58 failed to grow on either medium, 8 grew only on GGM, and none grew on GGM Pro⁻ only. Of the 8 strains growing only on GGM, 6 (75 per cent) gave a negative result in the transformation assay. All of the 18 strains that grew on both media gave a positive transformation result.

Sensitivity of transformation assay

The dense suspension of N. gonorrhoeae strain 9 contained 1.7×10^{10} c.f.u./ml and gave a positive transformation result up to a dilution of 10^6 . Because 0.1 ml of this suspension was placed in 0.9 ml of the SDS solution, the crude DNA preparation was obtained from cells at a concentration of 1.7×10^9 c.f.u.. Thus, 0.04 ml of the most concentrated sample of DNA tested in the transformation assay was derived from 6.8×10^7 c.f.u. and 0.04 ml of the maximum dilution that gave a positive result corresponded to 68 c.f.u..

The dense suspension of N. meningitidis serogroup B contained 4.7×10^9 c.f.u./ml and gave a positive transformation result up to a dilution of 10^2 . Because 0.1 ml of this suspension was placed in 0.9 ml of the SDS solution, the crude

DNA preparation was obtained from cells at a concentration of 4.7×10^8 c.f.u.. Thus, 0.04 ml of the most concentrated sample of DNA tested in the transformation assay corresponded to 1.9×10^7 c.f.u. and the maximum dilution that gave a positive result corresponded to 1.9×10^5 c.f.u..

Storage of test organisms on swabs

Swabs bearing test suspensions stored at 4°C and room temperature gave a positive result when tested at intervals up to 68 days. The effect of longer storage was not tested.

Transformation results with 42 urethral swab specimens from men

A diagnosis of gonorrhoea was made in 38 patients: 36 men had positive cultures and two had negative cultures but positive smears. Of these 38 patients, 14 (36.8 per cent) gave a positive transformation result. A positive transformation result was also obtained in one patient in whom there was no microbiological evidence of gonorrhoea. This patient was, however, a contact of a known case of gonorrhoea and was receiving treatment with ampicillin on epidemiological grounds at the time of the microbiological tests. Three patients diagnosed as non-gonococcal urethritis each had a negative transformation result.

Transformation results with cervical swabs from 23 contacts
of men with gonorrhoea

Eighteen (78.3 per cent) of the 23 contacts had microbiological evidence of gonorrhoea: 17 patients yielded positive cervical cultures and one patient, although culture-negative was smear-positive. Of the 18 patients with gonorrhoea, six (33.3 per cent) gave a positive transformation result. Specimens from all five patients in whom there was no microbiological evidence of gonorrhoea gave negative transformation results.

2. DETECTION OF GONOCOCCAL ANTIGENS BY THE ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Purified gonococcal LPS

The yield of freeze-dried LPS from 54.23g of cell paste of N. gonorrhoeae strain 9 was 1.16g.

Carbohydrate and protein estimation of the LPS preparation

The LPS extracted from N. gonorrhoeae strain 9 consisted predominantly of carbohydrate with a low content of protein. The ratio of carbohydrate to protein was approximately 15 to 1.

Carbohydrate and protein estimation of the outer membrane (OM) complex preparations

The OM complex preparations consisted predominantly of protein with a low content of carbohydrate. The ratios of protein to carbohydrate were within the range of 8 to 1 and 12 to 1.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The results of SDS-PAGE of OM complex preparations are shown in Figs. 7 and 8.

When the gels of the gonococcal OM complex preparations were stained with Coomassie brilliant blue, there were several

polypeptide bands common to all strains examined.

The Molecular Weight standards comprised four polypeptide bands corresponding to molecular weights of 14 300, 28 600, 42 900, and 57 200 as indicated by the manufacturer. The relative mobility of each polypeptide band was calculated according to the following formula:

$$\text{Percentage relative mobility} = \frac{\text{distance moved by band}}{\text{distance moved by bromophenol blue}} \times 100$$

A standard graph was drawn of relative mobility against molecular weight.

As determined by reference to the standard graph, most strains exhibited a major polypeptide band corresponding to an approximate molecular weight of 37 000; other densely staining bands corresponded to approximate molecular weights of 51 000 and 80 000.

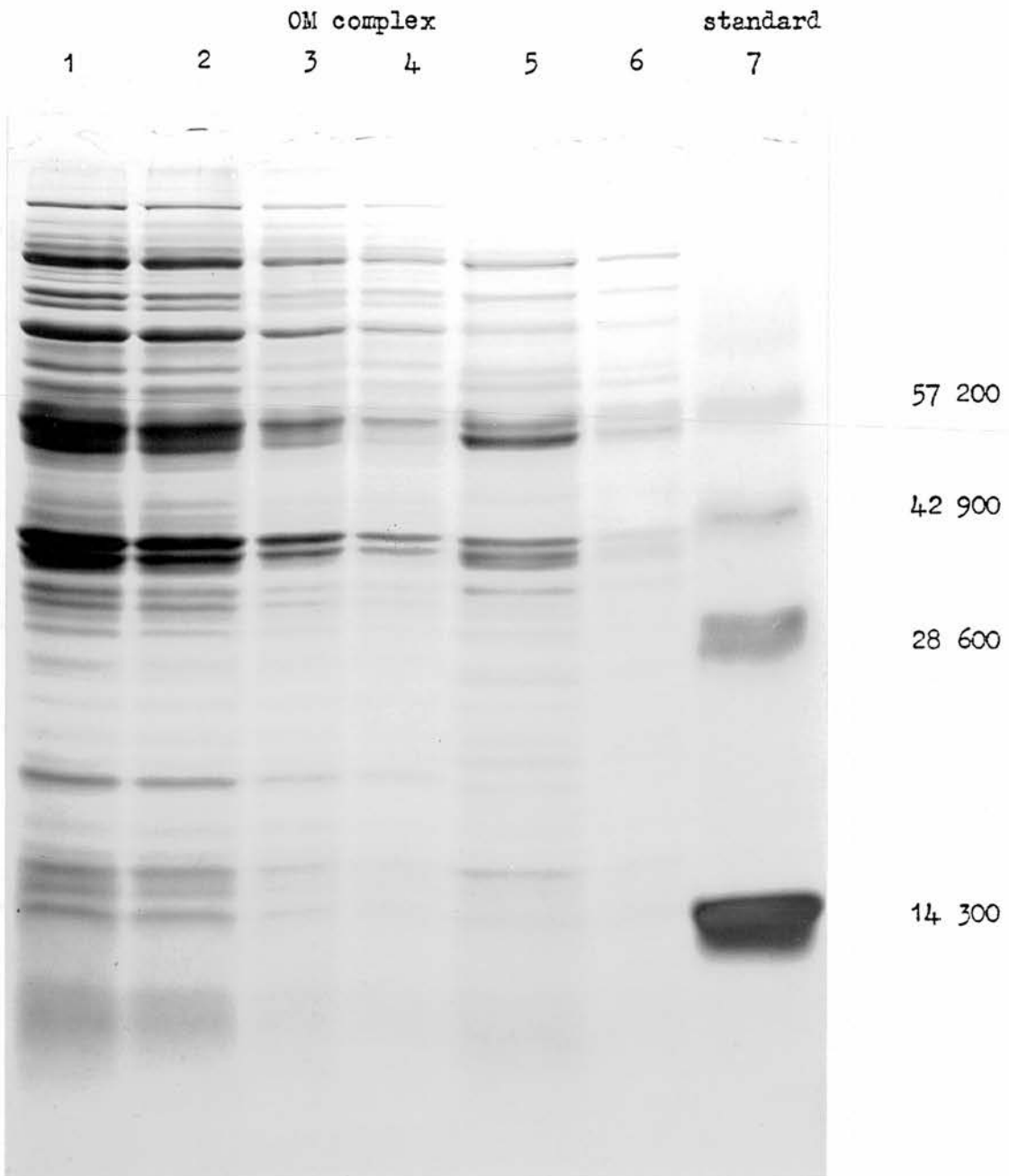


Fig. 7 SDS-PAGE of OM complex preparations from *N. gonorrhoeae* strains 9 and 82409

The gel was stained for protein with Coomassie brilliant blue. Profiles 1 to 4 represent OM complex from strain 9, containing 200, 100, 50, and 10 µg protein respectively. Profiles 5 and 6 represent OM complex from strain 82409, containing 50 and 10 µg protein respectively. Profile 7 contains the standard.

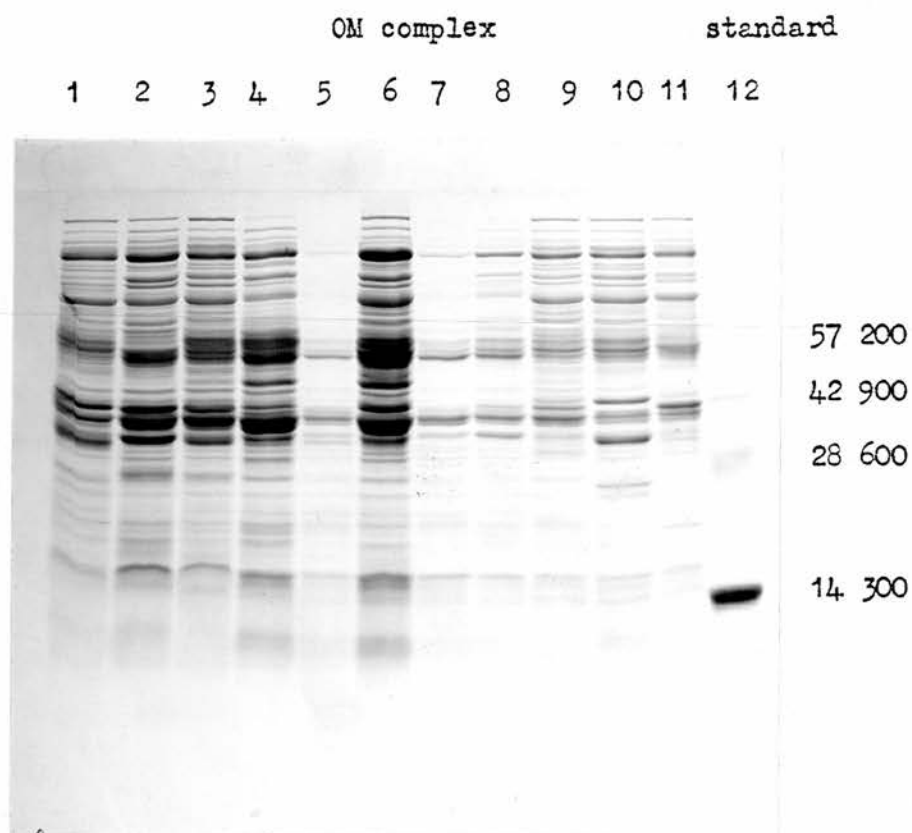


Fig. 8 SDS-PAGE of OM complex preparations from ten gonococcal clinical isolates and *N. gonorrhoeae* strain 9

The gel was stained for protein with Coomassie brilliant blue. Profiles 1 to 10 represent OM complex from ten clinical isolates of *N. gonorrhoeae*.

Profile 11 represents OM complex from strain 9.

All the samples examined contained 50 µg protein.

Profile 12 contains the standard.

2.1 Direct ELISA

Reading of ELISA results

The extinction values obtained with the negative controls varied within the range of 0.1 to 0.45.

In order to obtain a distinct 'cut off' point, a value of 0.9 was chosen as the end-point.

Antibody titres are expressed as the highest serum dilution at which the above end-point was given.

Microtitre plates coated with LPS (Fig. 2. p.108)

When LPS was used at the starting concentration of 10mg/ml, wells were coated with amounts of LPS ranging from 500 µg down to 0.24 µg. With conjugate at dilutions of 1 in 200 and 400, the highest extinction values corresponded to 15.6 µg of LPS and the end-points occurred with 0.24 µg of the antigen. With conjugate at dilutions of 1 in 800 and 1600, the highest extinction values corresponded to 62.5 µg of LPS and the respective end-points occurred with 15.6 and 31.2 µg of the antigen.

When LPS was used at the starting concentration of 1mg/ml, wells were coated with amounts of LPS ranging from 50 µg down to 0.02 µg. With conjugate at dilutions of 1 in 200 and 400, the highest extinction values corresponded to 12.5 µg of LPS and the end-points occurred with 0.19 µg of the antigen. With conjugate at dilutions of 1 in 800 and 1600,

the highest extinction values corresponded to 25 μ g of LPS and the end-points occurred with 0.78 μ g of the antigen.

Accordingly, conjugate at a dilution of 1 in 400 was thought to be suitable for the assay.

Antibody titres of rabbit antisera with purified gonococcal LPS as antigen (Fig. 3, p.109)

When the antibody titres of rabbit anti-strain 9 and anti-strain 82409 were determined with wells coated with 25, 12.5, 6.25, and 3.12 μ g of LPS, the following results were obtained:

LPS (μ g)	<u>Antibody titres</u>	
	anti-strain 9	anti-strain 82409
25	625	125
12.5	625	25
6.25	125	25
3.12	125	25

The highest antibody titres of anti-strain 9 and anti-strain 82409 were 625 and 125, respectively. These were lower than the corresponding antibody titres obtained with the gonococcal complement-fixation test (GCFT) which were 1024 and 256, respectively.

Antibody titres of rabbit antisera using whole cell (GCFT)
antigen in ELISA (Fig. 4, p.110)

Using the GCFT antigen prepared from N. gonorrhoeae strain 9 to coat the wells, the highest antibody titres of the rabbit anti-strain 9 and anti-strain 82409 were 625 and 125, respectively. These were lower than the corresponding antibody titres obtained with the GCFT.

Antibody titres of rabbit antisera with homologous OM complex
as antigen (Fig. 5, p.110)

The antibody titres of the rabbit anti-strain 9 and anti-strain 82409 sera were determined with wells coated with homologous OM complex corresponding to 5, 1, 0.2, and 0.04 μ g protein; the following results were obtained:

Protein (μ g)	<u>Antibody titres</u>	
	anti-strain 9	anti-strain 82409
5	15625	3125
1	15625	625
0.2	3125	125
0.04	125	25

When the wells were coated with N. gonorrhoeae strain 9 OM complex corresponding to 1 and 5 μ g protein, the assay

was 15.3 times more sensitive than GCFT.

When the wells were coated with N. gonorrhoeae strain 82409 OM complex corresponding to 5 μ g protein, the assay was 12.2 times more sensitive than GCFT.

Effect of OM complex protein on extinction

The results of the assay are presented in Fig. 9.

The extinction values increased with the amount of OM complex protein coating the wells, and reached a maximum at 0.78 μ g protein. Further increase in antigen beyond this point caused no further increase in the amount of bound antibody.

To allow as much as possible specific antigen-antibody binding to occur, an excess of antigens must be adsorbed in the wells (Brodeur et al., 1978; Glynn and Ison, 1978). A ten-fold dilution of the OM complex which corresponds to 5 μ g protein was chosen for subsequent experiments; this allowed economic use of the antigen, at a level well within the plateau.

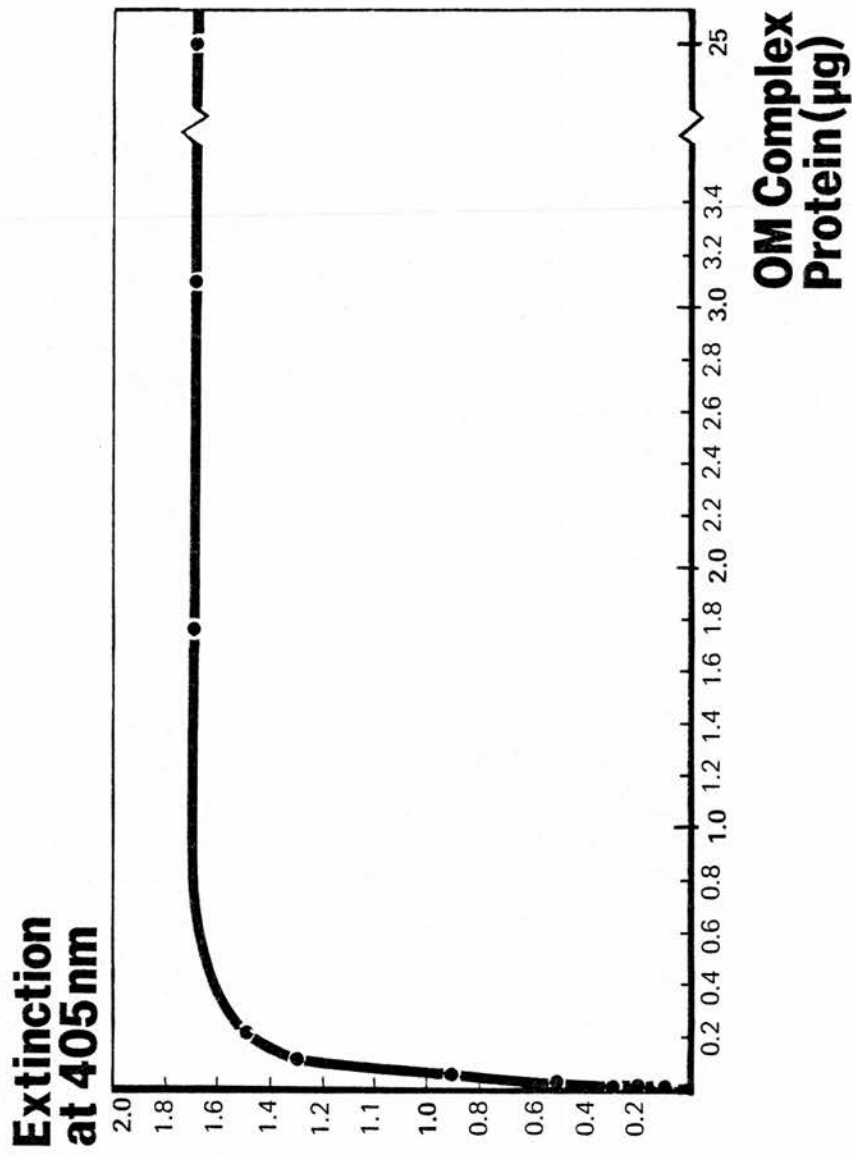


Fig. 2 Effect of OM complex protein on extinction.

Antibody titres of rabbit antisera tested against homologous and heterologous OM complex preparations

The rabbit antisera raised against N. gonorrhoeae strains 9 and 82409 tested against homologous OM complex had antibody titres of 15625 and 3125, respectively. When rabbit anti-strain 9 serum was tested against OM complex from N. gonorrhoeae strain 82409, its antibody titre remained unchanged. Similarly, the antibody titre of the rabbit anti-strain 82409 serum remained the same when tested against OM complex from N. gonorrhoeae strain 9.

The higher antibody titres obtained with the antiserum raised against strain 9, with both homologous or heterologous antigen, prompted its use in subsequent experiments.

ELISA-inhibition with purified LPS

When undiluted and a ten-fold dilution of the rabbit anti-strain 9 serum were preincubated with an equal volume of LPS (1mg/ml), the antibody titres were identical to those obtained with rabbit antiserum treated in the same manner except for the addition of an equal volume of PBS instead of LPS.

It would appear from this experiment that the antibody raised against N. gonorrhoeae strain 9 did not possess significant activity against LPS.

ELISA-inhibition with homologous OM complex

Undiluted and a ten-fold dilution of rabbit anti-strain 9 serum were preincubated with homologous OM complex or PBS. The antibody titres determined after preincubation indicated the greater decrease in the activity of antiserum when it was used at a dilution of 1 in 10. This dilution of antiserum was used in subsequent ELISA-inhibition experiments.

Antibody titres of rabbit antiserum obtained with various OM complex preparations as antigen

When the antibody titre of rabbit anti-strain 9 serum was determined with wells coated with homologous or heterologous OM complex, the following results were obtained:

Source of OM complex	Antibody titre of anti-strain 9 serum	Percentage activity of antiserum relative to antibody titre with strain 9 antigen
<u>N. gonorrhoeae</u> strain 9	12 800	100
<u>N. gonorrhoeae</u> (clinical isolate 1)	12 800	100
" " 2	12 800	100
" " 3	6 400	50
" " 4	3 200	25
" " 5	6 400	50
" " 6	6 400	50
" " 7	12 800	100
" " 8	12 800	100
" " 9	6 400	50
" " 10	6 400	50
<u>N. meningitidis</u> serogroup B	3 200	25
<u>N. lactamica</u>	1 600	12.5
<u>N. perflava</u>	1 600	12.5
<u>N. pharyngis</u>	800	6.25
<u>S. agalactiae</u>	-	0
<u>B. bivius</u>	-	0
<u>E. coli</u>	-	0

If 100 per cent activity corresponded to the antibody titre obtained with homologous OM complex, 25 per cent of that activity was displayed when the antiserum was tested against OM complex from N. meningitidis, and 6.25 to 12.5 per cent of that activity was displayed against the OM complex preparations from non-gonococcal neisseriae.

Specificity of the assay by ELISA-inhibition

Preincubation of the antiserum with homologous OM complex resulted in total inhibition of the assay.

With reference to antiserum preincubated with PBS, inhibition of the assay occurred when antiserum was preincubated with each of the OM complex preparations from ten clinical isolates of N. gonorrhoeae; this was indicated by a two to four-fold decrease of the antibody titre when compared with that of antiserum preincubated with PBS.

Preincubation with OM complex from N. meningitidis, N. lactamica, N. perflava, N. pharyngis, S. agalactiae, B. bivirus, and E. coli had no effect on the assay; the antibody titres were the same as that obtained with antiserum preincubated with PBS.

2.2 Indirect sandwich ELISA for the detection of

N. gonorrhoeae

Reading of indirect sandwich ELISA results

The extinction values obtained with the negative controls varied within the range of 0.35 to 0.47.

In order to obtain a distinct 'cut-off' point, a value of 1.0 was chosen as the end-point.

Dilutions of mouse and rabbit antisera to be used (Fig. 6, p.116)

When three sets of polystyrene balls were coated with 1 in 100, 200, and 300 dilutions of mouse antiserum for antigen capture, and the assay performed with dilutions of rabbit antiserum of 1 in 2500 and 5000, the minimum amounts of homologous OM complex protein detected were as represented below (figures shown are expressed in μg):

Rabbit antiserum	<u>Mouse antiserum</u>		
	1 in 100	200	300
1 in 2500	0.048	0.048	0.048
5000	0.048	0.048	0.096

Dilutions of mouse and rabbit antisera of 1 in 300 and 1 in 2500, respectively, were chosen for subsequent experiments; this allowed economic use of the mouse antiserum.

Detection of OM complex from ten clinical isolates of *N. gonorrhoeae*
and organisms other than gonococci

With mouse anti-strain 9 and rabbit anti-strain 9 sera at dilutions of 1 in 300 and 2500 respectively, the minimum amounts of heterologous OM complex protein detectable were determined; the following results were obtained:

Source of OM complex	Minimum amount of OM complex protein (μ g) detectable
<u><i>N. gonorrhoeae</i></u> strain 9	0.046
<u><i>N. gonorrhoeae</i></u> (clinical isolate 1)	0.046
" " 2	0.046
" " 3	0.046
" " 4	0.046
" " 5	0.046
" " 6	0.092
" " 7	0.046
" " 8	0.046
" " 9	0.092
" " 10	0.046
<u><i>N. meningitidis</i></u>	0.375
<u><i>S. agalactiae</i></u>	not detectable
<u><i>B. bivius</i></u>	"
<u><i>E. coli</i></u>	"

Detection of whole cell antigen as a preliminary to investigating clinical specimens

The suspension of N. gonorrhoeae strain 9 contained 4.9×10^7 c.f.u./ml. Therefore, 0.9 ml of this suspension contained 4.4×10^7 c.f.u.; when this was made up to 2 ml with 100 μ l of the 10x concentrated buffer and 1 ml of sputolysin, the suspension contained 2.2×10^7 c.f.u./ml. Since 300 μ l volumes from serial ten-fold dilutions of this suspension were used in the assay, and a positive result obtained up to a dilution of 10^3 , the minimum number of organisms detectable corresponded to 6.6×10^3 c.f.u..

3. DETECTION OF GONOCOCCAL COMPONENTS WITH COMMERCIALY AVAILABLE REAGENTS

3.1 Detection of endotoxin in clinical material by the Limulus lysate assay

Culture and microscopy

The results of culture and microscopy for N. gonorrhoeae are shown in relation to Limulus lysate assay results in Table 1. The Limulus assay was positive in 19 (28.8 per cent) of the 66 patients investigated. The test was reactive in 15 (62.5 per cent) of the 24 patients with cervical gonorrhoea (positive smear and/or culture) compared with only four (9.5 per cent) of the 42 patients without any microbiological evidence of gonorrhoea. This difference is statistically highly significant (χ^2 18.4; $P < 0.001$). When secretions from seven of the nine patients with gonorrhoea but negative Limulus assay results at a dilution of 1 in 100 were re-tested at 1 in 50, two gave a positive result; this increases the Limulus-positive-reactor rate to 17 (70.8 per cent) of 24 infected patients.

Table 1

Results of conventional microbiological investigations for *Neisseria gonorrhoeae* and limulus lysate assay for endotoxin in cervical secretions from 66 women

Pattern of microbiological results for <i>N. gonorrhoeae</i>	No. of patients with each pattern of results	No. limulus positive	No. limulus negative
Culture + Smear +	15	11	4
Culture + Smear -	6	3	3
Culture - Smear +	3	1	2
Culture - Smear -	42	4	38
Total	66	19	47

+ = positive; - = negative

Presenting diagnoses

The presenting diagnoses (multiple in a few cases) in the 42 patients in whom there was no microbiological evidence of gonorrhoea were as follows: Chlamydia trachomatis infection (eight); trichomoniasis (ten); candidiasis (eight); warts (five); no infection detected (nine). The remaining eight patients, all with negative Limulus assay results, had been treated for gonorrhoea within the preceding three months (range 1 week to 3 months).

The Limulus assay was positive in one patient who was a contact of gonorrhoea, one patient from whom C. trachomatis was isolated, one patient with warts, and one patient in whom no abnormality was detected. If the patient who was a contact of gonorrhoea is excluded, the presumed false positive rate is reduced to 7.3 per cent: on re-testing secretions from two of the three remaining patients at a dilution of 1 in 200, both were negative reducing the false positive rate to 2.4 per cent.

Mean protein concentration

The mean protein concentration in the secretions from four patients with gonorrhoea who gave negative Limulus results was 0.42 mg/ml compared with a mean concentration of 1.5 mg/ml in the secretions from five patients with gonorrhoea who gave positive Limulus results. The difference between the two means is statistically significant (t 10.12; $P < 0.001$).

3.2 Coagglutination test

Assessment of sensitivity of the test procedure with *N. gonorrhoeae* strain 9 and a strain of *N. meningitidis* serogroup B

The suspensions of *N. gonorrhoeae* strain 9 and a strain of *N. meningitidis* serogroup B contained 7.3×10^8 c.f.u./ml and 1.25×10^9 c.f.u./ml, respectively.

The coagglutination end-points with *N. gonorrhoeae* strain 9 and a strain of *N. meningitidis* serogroup B, utilizing 40 μ l volumes, were at the dilutions of 1 in 32 and 1 in 2 respectively. With reference to the viable counts obtained, 40 μ l of the suspension of *N. gonorrhoeae* strain 9 at a dilution of 1 in 32 contained 9.1×10^5 c.f.u.; 40 μ l of the suspension of *N. meningitidis* at a dilution of 1 in 2 contained 2.5×10^7 c.f.u..

The results indicated that with *N. gonorrhoeae* strain 9, the test was 27.4 times more sensitive than with a strain of *N. meningitidis* serogroup B.

4. MICROFLORA OF CERVIX AND VAGINA OF PATIENTS WITH AND WITHOUT GONORRHOEA

The numbers of organisms isolated from the cervix and vagina of 20 patients with gonorrhoea, and 17 patients without, are shown in tables 2 and 3.

The frequency of isolation of each organism from the cervix and vagina of 18 patients with and 15 patients without gonorrhoea is shown in tables 4 and 5: quantitative cultures from one patient were spoiled by overgrowth with Proteus, and only quantitative gonococcal cultures were performed with aspirates from one patient with and two patients without gonorrhoea.

Table 2 (see over)

Cervical and vaginal microflora of 20 patients with gonorrhoea

Results are expressed as total c.f.u. aspirated in a 1ml volume from the cervix, and a 5ml volume from the vagina.

* N. gonorrhoeae was isolated from repeat cultures taken one week later, quantitative counts were not repeated; ** cultures were overgrown with Proteus; *** Clostridium sp. was isolated from the vagina (9.0×10^5 c.f.u./5ml). No haemolytic streptococci, T. vaginalis, or C. albicans were isolated.

Cervicitis was diagnosed when the cervix was oedematous and reddened, and mucopus exuded from the external os.

Key: C = cervix; V = vagina; N. gon. = N. gonorrhoeae; Staph. = Staphylococcus; Ent. = enterococci; Lact. = lactobacilli; Diph. = diphtheroids; C. vag. = Corynebacterium vaginale; Anaer. = anaerobes; S = anaerobic streptococci; B = Bacteroides bivius; Chlam. = Chlamydia; U. urea. = Ureaplasma urealyticum.

Patients	Site	Condition of cervix	N. gon.	Staph.	Ent.	Lact.	Diph.	C. vag.	Anaer.	Chlam.	U. urea.
1	C	Cervicitis	2.4×10^4	1.0×10^2	1.0×10^2	2.0×10^6					10^5
	V		3.1×10^4	3.0×10^3	4.5×10^4	2.4×10^8			2.5×10^{3S}		$\gg 5.0 \times 10^{10}$
2	C	Cervicitis	6.6×10^5		1.0×10^4			5.0×10^7			$\gg 10^{10}$
	V		1.4×10^6	2.3×10^5	1.1×10^6			$\gg 5.0 \times 10^8$			$\gg 5.0 \times 10^{10}$
10	C	Healthy	5.0×10^4			1.0×10^8					10^3
	V		1.1×10^4	3.2×10^4	2.5×10^4	$\gg 5.0 \times 10^8$					5.0×10^1
11	C	Cervicitis	1.4×10^4	1.0×10^2			4.0×10^4				10^7
	V		3.0×10^3	4.0×10^3			1.0×10^7				$\gg 5.0 \times 10^{10}$
13	C	Cervicitis	2.8×10^4			1.0×10^8				+	10^7
	V		1.6×10^4	1.5×10^4		$\gg 5.0 \times 10^8$	5.0×10^8		1.5×10^{3B}		5.0×10^5
14	C	Cervical polyp	1.5×10^5	1.0×10^2		2.0×10^3		4.0×10^6			
	V		8.5×10^4	1.2×10^4		2.5×10^5		$\gg 5.0 \times 10^8$			
15	C	Cervicitis	2.3×10^6								
	V		5.2×10^6	1.5×10^3							
17	C	Cervicitis	9.0×10^3	4.0×10^3		4.8×10^6	8.0×10^4				10^4
	V		1.5×10^3	5.0×10^5		$\gg 5.0 \times 10^8$	1.5×10^6				5.0×10^6
18	C	Cervicitis	1.8×10^5				$\gg 1.0 \times 10^8$		1.0×10^{8BS}		10^3
	V		1.3×10^4	4.5×10^3			$\gg 5.0 \times 10^8$		$\gg 5.0 \times 10^{8BS}$		5.0×10^3
19	C	Healthy	2.8×10^5	1.0×10^3		$\gg 1.0 \times 10^8$	4.0×10^3				10^6
	V		1.9×10^4	1.4×10^5		$\gg 5.0 \times 10^8$	1.2×10^4				$\gg 5.0 \times 10^{10}$
22	C	Cervicitis	8.0×10^6			1.0×10^6					
	V		2.0×10^5			$\gg 5.0 \times 10^8$					
24	C	Cervicitis	9.0×10^3	1.0×10^2		$\gg 1.0 \times 10^8$					$\gg 10^{10}$
	V		$\langle 5.0 \times 10^2$	5.0×10^3	4.4×10^4	$\gg 5.0 \times 10^8$					5.0×10^7
26*	C	Cervicitis	$\langle 10^2$	1.6×10^3		1.0×10^8	9.0×10^3			+	10^3
	V		$\langle 5.0 \times 10^2$	1.5×10^5		$\gg 5.0 \times 10^8$	5.5×10^5				5.0×10^7
29	C	Cervicitis	5.8×10^6	1.2×10^4			5.8×10^4				10^5
	V		8.5×10^4	5.0×10^4			6.5×10^4				5.0×10^5
30**	C	Healthy	8.0×10^4	2.4×10^3							10^5
	V		3.0×10^4	2.4×10^4							5.0×10^7
31***	C	Healthy	3.7×10^5	1.0×10^3	1.4×10^3		5.8×10^7			+	$\gg 10^{10}$
	V		4.5×10^3	1.5×10^4	5.0×10^4	2.4×10^4	$\gg 5.0 \times 10^8$				$\gg 5.0 \times 10^{10}$
32	C	Healthy	1.4×10^6	5.0×10^3		1.0×10^6	3.4×10^6				10^4
	V		$\langle 5.0 \times 10^2$	1.4×10^5		2.0×10^8	$\gg 5.0 \times 10^8$				5.0×10^3
33	C	Healthy	7.0×10^3	3.0×10^2			2.0×10^4			+	10^2
	V		5.0×10^2	4.5×10^4			4.0×10^6				5.0×10^4
34	C	Ectropian	1.4×10^4	2.0×10^3			5.5×10^6				10^3
	V		7.0×10^4	3.0×10^5			4.7×10^8				5.0×10^6
36	C	Cervicitis	5.4×10^3							+	
	V		1.0×10^3								

Table 3 (see over)

Cervical and vaginal microflora of 17 patients without gonorrhoea

Results are expressed as total c.f.u. aspirated in a 1ml volume from the cervix, and a 5ml volume from the vagina.

* E. coli was isolated from the cervix (8.0×10^2 c.f.u./ml) and vagina (1.5×10^5 c.f.u./5ml).

Cervicitis was diagnosed when the cervix was oedematous and reddened, and mucopus exuded from the external os.

Key: C = cervix; V = vagina; Staph. = Staphylococcus; Haem. strept. = haemolytic streptococci; Gr. = group; Ent. = enterococci; Lact. = Lactobacilli; Diph. = diphtheroids; Anaer. = anaerobes; S = anaerobic streptococci; B = Bacteroides bivius; C. alb. = Candida albicans; T. vag. = Trichomonas vaginalis; Chlam. = Chlamydia; U. urea. = Ureaplasma urealyticum.

[illegible]

Table 4

Microbial isolates from cervical cultures of 18 patients with gonorrhoea and 15 patients without gonorrhoea

Organisms	<u>Patients with gonorrhoea</u>		<u>Patients without gonorrhoea</u>	
	number	per cent	number	per cent
<u>N. gonorrhoeae</u>	17	94.4*	0	0
<u>Staphylococcus</u>	12	66.6	7	46.6
Haemolytic streptococci	0	0	2	13.3
Enterococci	3	16.6	2	13.3
Anaerobic streptococci	1	5.5	1	6.6
Lactobacilli	10	55.5	12	80
Diphtheroids	10	55.5	8	53.3
<u>C. vaginale</u>	2	11.1	0	0
<u>Clostridium</u>	0	0	0	0
<u>B. bivius</u>	1	5.5	2	13.3
<u>E. coli</u>	0	0	1	6.6
<u>C. albicans</u>	0	0	1	6.6
<u>Chlamydia</u>	4	22.2	1	6.6
<u>U. urealyticum</u>	15	83.3	12	80

* The patient with negative gonococcal cultures at the time of the quantitative examination gave positive cultures when tested one week later.

Proteus was isolated from one (5.2 per cent) of 19 patients with gonorrhoea; the resulting overgrowth rendered cultures for other organisms invalid. The results are calculated on the basis of 18 patients with valid quantitative results.

Table 5

Microbial isolates from vaginal cultures of 18 patients with gonorrhoea and 15 patients without gonorrhoea

Organisms	<u>Patients with gonorrhoea</u>		<u>Patients without gonorrhoea</u>	
	number	per cent	number	per cent
<u>N. gonorrhoeae</u>	15	83.3	0	0
<u>Staphylococcus</u>	17	94.4	13	86.6
Haemolytic streptococci	0	0	4	26.6
Enterococci	5	27.7	4	26.6
Anaerobic streptococci	2	11.1	2	13.3
Lactobacilli	11	61.1	11	73.3
Diphtheroids	11	61.1	8	53.3
<u>C. vaginale</u>	2	11.1	0	0
<u>Clostridium</u>	1	5.5	0	0
<u>B. bivius</u>	2	11.1	2	13.3
<u>E. coli</u>	0	0	1	6.6
<u>C. albicans</u>	0	0	2	13.3
<u>T. vaginalis</u>	0	0	3	20
<u>U. urealyticum</u>	15	83.3	12	80

Proteus was isolated from one (5.2 per cent) of 19 patients with gonorrhoea; the resulting overgrowth rendered cultures for other organisms invalid. The results are calculated on the basis of 18 patients with valid quantitative results.

5. DETECTION OF GONOCOCCAL COMPONENTS IN THE CERVICAL AND VAGINAL SECRETIONS INVESTIGATED BACTERIOLOGICALLY

The results for the 20 patients with gonorrhoea are shown in table 6, and those for the 17 patients without gonorrhoea are shown in table 7.

Intracellular Gram-negative diplococci were observed in Gram stained smears of cervical exudate from 11 (55 per cent) of 20 patients with culture-proven gonorrhoea. Nineteen patients had positive routine cultures at the time aspirates were examined quantitatively; one patient yielded a positive cervical culture for N. gonorrhoeae at the second set of routine diagnostic tests repeated one week later.

5.1 Transformation assay

Of the 20 patients with gonorrhoea, 15 (75 per cent) gave a positive transformation result with cervical or vaginal aspirates; cervical aspirates were positive in 13 (65 per cent) and vaginal aspirates in 7 (35 per cent).

Of the 17 patients with no microbiological evidence of gonorrhoea, 2 (11.7 per cent) gave a positive transformation result. The cervical and vaginal aspirates were positive in one patient who was a contact of gonorrhoea; only the cervical aspirate was positive in the second patient. The condition diagnosed in both patients was trichomoniasis.

5.2 Indirect sandwich ELISA

Of the 20 patients with gonorrhoea, 12 (60 per cent) gave a positive ELISA result; cervical aspirates were positive in 11 (55 per cent) and vaginal aspirates in one (5 per cent).

Cervical aspirates from two (11.7 per cent) of the 17 patients with no microbiological evidence of gonorrhoea gave a positive ELISA result. The conditions diagnosed were urinary tract infection in one patient; non-specific genital infection, warts, and pubic lice in the second. Neither patient was a contact of gonorrhoea.

5.3 Limulus lysate assay

The test was reactive in 16 (84.2 per cent) of 19 patients with cervical gonorrhoea, compared with 3 (17.6 per cent) of 17 patients without any microbiological evidence of gonorrhoea.

When secretions from the three patients with gonorrhoea but negative Limulus assay results at a dilution of 1 in 100 were re-tested at 1 in 50, two gave a positive result; this increases the Limulus-positive-reactor rate to 18 (94.7 per cent) of 19 infected patients.

The Limulus assay was positive in three patients with no microbiological evidence of gonorrhoea; the presenting diagnoses in two of these patients were trichomoniasis and candidiasis respectively, and no abnormality was detected in the third patient who was a contact of gonorrhoea. On re-testing secretions from the three patients at a dilution of

1 in 200, all were negative reducing the false positive rate to 0.

When the secretions from the 16 patients which gave a positive Limulus assay result at a dilution of 1 in 100 were re-tested at 1 in 200, the Limulus-positive-reactor rate was reduced to 13 (68.4 per cent) of 19 infected patients.

5.4 Coagglutination test

All the cervical secretions from the 20 patients with gonorrhoea and the 17 patients without gonorrhoea gave a negative coagglutination result.

Table 6 (see over)

Results of tests to detect gonococcal components in 20 patients
with gonococcal infection confirmed by conventional microbiological
methods

Quantitative results for N. gonorrhoeae are expressed as total c.f.u. aspirated in a 1ml volume from the cervix, and a 5ml volume from the vagina.

Key: C = cervix; V = vagina.

Patients	Site	<u>N. gonorrhoeae</u> isolated	Smear	Transformation assay	Indirect sandwich ELISA	<u>Limulus</u> assay	Coagglutination test
1	C	2.4×10^4	—	+	—	—	—
	V	3.1×10^4		+	+		
2	C	6.6×10^5	+	+	+	+	—
	V	1.4×10^6		+	—		
10	C	5.0×10^4	—	—	—	+	—
	V	1.1×10^4		—	—		
11	C	1.4×10^4	+	—	+	+	—
	V	3.0×10^3		+	—		
13	C	2.8×10^4	+	+	+	+	—
	V	1.6×10^4		—	—		
14	C	1.5×10^5	—	+	+	not done	—
	V	8.5×10^4		+	—		
15	C	2.3×10^6	+	+	+	+	—
	V	5.2×10^6		—	—		
17	C	9.0×10^3	—	+	—	—	—
	V	1.5×10^3		—	—		
18	C	1.8×10^5	—	+	+	+	—
	V	1.3×10^4		—	—		
19	C	2.8×10^5	—	—	—	+	—
	V	1.9×10^4		—	—		
22	C	8.0×10^6	+	+	+	—	—
	V	2.0×10^5		—	—		
24	C	9.0×10^3	—	+	+	+	—
	V	$< 5.0 \times 10^2$		+	—		
26	C	$< 10^2$	—	+	+	+	—
	V	$< 5.0 \times 10^2$		+	—		
29	C	5.8×10^6	+	+	—	+	—
	V	8.5×10^4		—	—		
30	C	8.0×10^4	+	+	—	+	—
	V	3.0×10^4		—	—		
31	C	3.7×10^5	+	—	—	+	—
	V	4.5×10^3		—	—		
32	C	1.4×10^6	—	—	—	+	—
	V	$< 5.0 \times 10^2$		—	—		
33	C	7.0×10^3	+	—	+	+	—
	V	5.0×10^2		+	—		
34	C	1.4×10^4	+	—	—	+	—
	V	7.0×10^4		—	—		
36	C	5.4×10^3	+	+	+	+	—
	V	1.0×10^3		—	—		

Table 7 (see over)

Results of tests to detect gonococcal components in 17 patients
in whom gonococcal infection was excluded by conventional
microbiological methods

Key: C = cervix; V = vagina.

Patients	Diagnosis	Site	Smear	Transformation assay	Indirect sandwich ELISA	Limulus assay	Coagglutination test
3	Warts	C V	-	- -	- -	-	-
4	Urinary tract infection	C V	-	- -	+ -	-	-
5	Trichomoniasis	C V	-	+ -	- -	-	-
6	No abnormality detected	C V	-	- -	- -	-	-
7	Urinary tract infection	C V	-	- -	- -	-	-
8	Trichomoniasis	C V	-	- -	- -	+	-
9	No abnormality detected	C V	-	- -	- -	-	-
12	Non specific genital infection Warts Pubic lice	C V	-	- -	+ -	-	-
16	No abnormality detected	C V	-	- -	- -	-	-
20	Candidiasis	C V	-	- -	- -	+	-
21	No abnormality detected	C V	-	- -	- -	-	-
23	No abnormality detected Contact of gonorrhoea	C V	-	- -	- -	-	-
25	Candidiasis	C V	-	- -	- -	-	-
27	No abnormality detected Contact of gonorrhoea	C V	-	- -	- -	-	-
28	Trichomoniasis Contact of gonorrhoea	C V	-	+ +	- -	-	-
35	No abnormality detected Contact of gonorrhoea	C V	-	- -	- -	+	-
37	Trichomoniasis Non specific genital infection Contact of gonorrhoea	C V	-	- -	- -	-	-

DISCUSSION

1. TRANSFORMATION STUDIES

Preliminary transformation experiments

These results confirm the findings of Janik et al. (1976) that genetic transformation can be used as a tool for the identification of N. gonorrhoeae. Although a different strain was used as recipient, the overall sensitivity of the assay is comparable. Janik et al. (1976) found that approximately 50 c.f.u. of donor cells were required to give a positive result, compared with approximately 70 c.f.u. in the present study with N. gonorrhoeae strain F62. As Janik et al. (1976) found, colony-type 2 was more efficient than colony-type 3 in the uptake of DNA.

With the exception of N. meningitidis and N. lactamica, the transformation assay is specific for N. gonorrhoeae. A positive result was obtained with all 12 clinical meningococcal isolates and the stock cultures of the different meningococcal serogroups. These results are also similar to those of Janik et al. (1976) who found that DNA preparations from only a few neisseriae other than the gonococcus were able to transform their proline auxotrophs; these preparations were unable to transform a uracil and arginine auxotroph in the standard transformation assay conditions. The lack of specificity with regard to N. meningitidis would not seriously reduce the value of the assay in detecting ano-genital gonorrhoea because meningococci are relatively rare in the urogenital tract and anal canal (Givan, Thomas and Johnston, 1977; Blackwell,

Young and Bain, 1978), and the number required to give a positive transformation assay is approximately 1000-fold higher than when gonococci are used. Nevertheless, special care must be taken with specimens from homosexual men. In one locality, Chapel, Gatewood and Keane (1977) isolated N. meningitidis from the oropharynx and anal canal of 32 (20.4 per cent) of 157 homosexual men (oropharynx, 17.8 per cent; anal canal, 4.5 per cent; urethra, 0); three other men had N. meningitidis cultured from both oropharynx and anal canal. Moreover, in view of the positive results given by meningococci, the genetic transformation assay described would not be suitable for diagnosing pharyngeal gonorrhoea.

The possible presence of naturally-occurring proline auxotrophs does not appear to limit the value of the assay greatly because approximately 90 per cent of the clinical isolates gave a positive transformation assay. In another locality, Bawdon, Juni and Britt (1977) reported that 97 per cent of 71 clinical isolates of N. gonorrhoeae gave a positive transformation result in an assay that used a uracil and arginine auxotroph. Proline requirement is probably the main reason for negative results in our survey because 6 (75 per cent) of 8 strains that failed to grow on GGM Pro⁻ gave negative results, whereas all of the 18 isolates that grew on both GGM and GGM Pro⁻ gave positive results. However the results with respect to the growth of isolates on GGM and GGM Pro⁻ do not seem to be very reliable because 58 (69 per cent) of 84 fresh clinical isolates failed to grow on both media; clinical isolates probably need to be adapted to growth in the

laboratory before giving reliable growth on a minimal medium such as GGM.

After prolonged storage of gonococci on swabs, a positive transformation result could still be obtained. Therefore the ability of the assay to detect non-viable gonococci would make this test of value in overcoming problems associated with the transport of specimens before cultivation by conventional methods.

Transformation assay with urethral and cervical swab specimens

The transformation assay was positive in 36.8 per cent of urethral swab specimens from men and 33.3 per cent of cervical swab specimens from women with untreated gonorrhoea. In contrast to these results Janik et al. (1976) obtained a positive transformation assay result with all 90 swab specimens confirmed by conventional identification methods; whether these swabs were obtained from men or women was not specified. Using swab specimens, the sensitivity of the assay was therefore surprisingly low in the present study. It was considered possible that the cotton wool was retaining the organisms which should be released in to the lysing solution for the preparation of crude DNA, and that another method of collecting specimens should improve the sensitivity of the assay. Unfortunately, the type of swab used by Janik et al. (1976) was not stated in their report.

From the patients with no microbiological evidence of gonorrhoea, only one swab specimen gave a positive transformation result; it was taken from a man who was a contact of a known case of gonorrhoea, and was receiving treatment on epidemiological grounds at the time of the microbiological tests. It is possible that DNA derived from non-viable gonococci was detected in this patient.

2. DETECTION OF GONOCOCCAL ANTIGENS BY THE ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Direct ELISA studies

The aim of these preliminary experiments was to determine the feasibility of using rabbit antiserum raised against whole untreated cells of N. gonorrhoeae to detect gonococcal antigens.

Cell surface antigen(s) detectable by the rabbit antiserum

In common with other Gram-negative bacteria, LPS is located on the outermost aspect of the outer membrane of N. gonorrhoeae (Johnston and Gotschlich, 1974; Wolf-Watz et al., 1975). However, our experiments showed that in spite of this, LPS is not a suitable antigen to detect with antisera raised against whole cells. In fact, when LPS from N. gonorrhoeae colony-type 3 was used to coat the microtitre wells, the highest antibody titres obtained with anti-strain 9 and anti-strain 82409 sera were respectively 1.6 and 2.0 times lower than the corresponding antibody titres obtained in the gonococcal complement fixation test (GCFT), in which whole cell N. gonorrhoeae strain 9 antigen was used. It was concluded that LPS was a poor immunogen in the rabbit and was not the main antigen to elicit an antibody response, and/or it bound poorly to polystyrene. Diena et al. (1978) have presented evidence to show that even R-type LPS is immunogenic in relatively minute quantities since immunized mice were protected against intracerebral challenge, and embryos obtained from immunized hens were protected against intravenous

challenge. Bactericidal and passive-protection antibody were present in a pool of sera obtained from the immunized mice; the sera of the embryos of immunized hens did not contain bactericidal antibody despite the fact that bactericidal antibody was present in the sera of immunized hens from which the embryos were obtained. This suggested to the authors that the protection transmitted to the embryos from immunized hens might be due to cellular rather than humoral factors.

In the present study, despite the fact that antiserum was raised against whole cells, the LPS component of the gonococci would have the opportunity of reacting with the immune system. However, as shown by Perry et al. (1977), LPS is a poor immunogen in many animals including the rabbit, and gives rise to a significant antibody production only in hens.

Recently, poor binding of LPS to polystyrene has been demonstrated by Ito et al. (1980) in ELISA experiments using ^{51}Cr radiolabelled LPS as antigen. Satisfactory adsorption was achieved when Mg^{++} was added to LPS. Subsequently, the antibody titres of three batches of antisera from rabbits immunized with boiled cells from three colony-type 2 gonococcal strains were determined with LPS, isolated from one of the strains used for immunization, as antigen. The antibody titre of the homologous antiserum was the highest (> 2048), while the antibody titres of the two heterologous antisera were relatively low (32 and 64). This suggested to the investigators that LPS from these gonococci were cross-reactive but contained different O-antigens. Therefore,

the main problems in using LPS as antigen are antigenic heterogeneity of O side-chains, poor binding to polystyrene, and relatively poor immunogenicity in the rabbit.

Poor binding was also the most likely explanation for the poor results obtained when whole cell (GCFT) antigen was used in the assay; the antibody titres remained lower than those obtained in the GCFT. Most antigens adhere to polystyrene surfaces by physical adsorption, and antigens used for ELISA are soluble (Bulletin of the World Health Organization, 1976). The GCFT antigen consisting primarily of whole cells was insoluble. The surface area of the whole cell antigen making contact with the polystyrene surface may have been relatively small in comparison with the size of the antigen, and was unable to withstand the washing stages during which the cells were probably washed off the polystyrene surface.

The most satisfactory results were obtained when OM complex rich in protein was used as antigen. SDS-polyacrylamide gel electrophoresis of gonococcal OM complex preparations demonstrated a major polypeptide band of molecular weight 37 000, corresponding to the protein I described by Heckels (1977). Heckels (1978) also investigated the topographical distribution of the OM protein antigens; autoradiography of an SDS-polyacrylamide gel of gonococci labelled with ^{125}I -lactoperoxidase revealed that protein I was always present on the cell surface, and was heavily labelled. This protein therefore constitutes a major surface antigen and confers serotype specificity: Johnston et al. (1976)

used OM complex to assign clinical isolates of N. gonorrhoeae in to 16 distinct serotypes. Results of SDS-polyacrylamide gel electrophoresis of OM complex preparations revealed that several polypeptide bands common to the strains of N. gonorrhoeae examined were present, and some of these were probably responsible for eliciting an antibody response in the rabbit. These findings suggested that antiserum raised against a single strain of N. gonorrhoeae should be able to cross-react with OM complex from a variety of gonococcal strains.

The direct ELISA results confirm the evidence presented by other investigators for adequate binding of protein to polystyrene (Brodeur et al., 1978; Glynn and Ison, 1978; Poxton, 1979; Young and Low, 1981). With homologous OM complex, the antibody titres of the anti-strain 9 and anti-strain 82409 rabbit sera were 15.3 and 12.2 times higher than when determined by GCFT. Since protein was the major component of the OM complex preparations, the high antibody titres obtained also indicated that OM protein was a major antigen which elicited an antibody response in the rabbit.

When the antibody titres of the two rabbit antisera were determined against their respective heterologous OM complex preparations, the antibody titres remained unchanged; that of the rabbit anti-strain 9 was higher with both homologous and heterologous (strain 82409) OM complex. Consequently, it was thought that the common gonococcal antigens are better exposed on the whole cells of N. gonorrhoeae strain 9, and that this provokes a more complete immune response in the immunized rabbit.

This is in agreement with the view that N. gonorrhoeae strain 9 possesses antigenic features that might be quantitatively or qualitatively specific to gonococci (O'Reilly et al., 1973). Accordingly, it was decided that rabbit antiserum raised against N. gonorrhoeae strain 9 is more suitable for the detection of gonococcal antigens.

ELISA-inhibition experiments

These experiments confirmed that OM complex protein is an important antigen. Preincubation of a ten-fold dilution of antiserum with homologous OM complex resulted in total inhibition of the assay, while preincubation with undiluted antiserum indicated that antibody was in excess with respect to antigen. Consequently, a ten-fold dilution of the rabbit antiserum was used in subsequent ELISA-inhibition experiments. In contrast, preincubation of homologous antiserum with LPS did not result in an apparent inhibition of the assay; this was not unexpected since OM complex used for coating the wells consisted primarily of protein.

Activity of antiserum with various heterologous OM complex preparations

The antibody titre obtained with OM complex from a strain of N. meningitidis serogroup B corresponded to the lowest antibody titre obtained with OM complex from one clinical isolate of N. gonorrhoeae. The activity displayed with OM complex from the commensal neisseriae tested was negligible, and none could be demonstrated with OM complex from S. agalactiae, B. bivius,

and E. coli. The specificity of the antiserum to N. gonorrhoeae employed in the assay is important in order to avoid interference by other organisms present in the uro-genital tract. As discussed earlier with regard to the transformation assay, the lack of specificity with N. meningitidis does not significantly limit the value of the assay when applied to cervical secretions.

ELISA-inhibition with various heterologous OM complex preparations

Inhibition of the assay was observed only with OM complex from ten clinical isolates of N. gonorrhoeae; none of the OM complex preparations from the non-gonococcal neisseriae resulted in inhibition. It was concluded that the antibodies to cross-reactive antigens did not constitute the major antibody response, since their level was low enough to remain undetected by ELISA-inhibition.

Accordingly, the antibody response was considered to be directed primarily towards OM antigens specific for N. gonorrhoeae, making the rabbit anti-strain 9 serum suitable for detecting gonococcal antigens.

Indirect sandwich ELISA

An indirect sandwich ELISA system described by Drow et al. (1979) for the detection of Haemophilus influenzae type b (HI_b) antigen in clinical specimens from patients with HI_b meningitis was found to be highly sensitive and specific. The assay reproducibly detected purified HI_b capsular antigen polyribose-phosphate at concentrations between 1 and 5 ng/ml. A total of

18 specimens (spinal fluid, 11; urine, 5; serum, 2) from 11 patients with culture-proven HI_b meningitis were assayed.

Testing both the undiluted and a five-fold dilution, the assay detected HI_b antigen in all 18 specimens, even when diluted five-fold. Following this report, the detection of the polysaccharide antigen of type 3 pneumococcus by the indirect sandwich ELISA was investigated (Drow and Manning, 1980). A positive assay was obtained routinely with specimens containing 10^6 c.f.u./ml of Streptococcus pneumoniae type 3. The assay was negative with K. pneumoniae, E. coli, H. influenzae, Ps. aeruginosa, group B streptococcus, and S. aureus suspended in PBS; the viable counts for these organisms were within the range of 2.0×10^8 and 5.0×10^8 c.f.u./ml.

In the present study, the results obtained compare favourably with the previous findings. Despite the lower sensitivity of our assay, as little as 46 ng OM protein could be detected. The sensitivity of the assay remained unchanged with eight of 10 OM complex preparations from clinical isolates of N. gonorrhoeae; with the two remaining OM complex preparations, 92 ng OM protein was the minimum amount which could be detected. The sensitivity of the assay decreased approximately eight-fold with OM complex from a strain of N. meningitidis serogroup B; the minimum amount of OM protein detected was 375 ng. The results for OM complex from S. agalactiae, B. bivius, and E. coli were in accordance with the previous findings by the direct ELISA; all three preparations gave negative results. Therefore the assay was highly specific for gonococcal antigens.

When the assay was performed with a simulated clinical specimen containing N. gonorrhoeae strain 9, a minimum of 6.6×10^3 c.f.u. (or 2.1×10^4 c.f.u./ml) could be detected.

The encouraging results obtained in the preliminary ELISA experiments justified further evaluation of the system in the detection of gonococcal antigens in clinical specimens from patients infected with N. gonorrhoeae.

3. PRELIMINARY EVALUATION OF THE LIMULUS LYSATE ASSAY APPLIED TO CERVICAL SECRETIONS

Although the number of infected patients was low, there was a highly statistically significant correlation ($P < 0.001$) between Limulus lysate assay results and conventional microbiological evidence of gonorrhoea. The Limulus assay was positive in only 62.5 per cent of women with cervical gonorrhoea and was less sensitive than Gram-staining which detected 75 per cent of infected women. However, the 62.5 per cent Limulus-positive-reactor rate is within the usual range (55-65 per cent) of sensitivity found by Gram-staining (Barlow *et al.*, 1976; Chipperfield and Catterall, 1976; Evans, 1976).

In the study of Spagna *et al.* (1979), the Limulus assay applied to urethral exudates from men was positive in all 73 culture-positive cases of gonorrhoea tested. Our finding that the Limulus-positive-reactor rate could be increased to 70.8 per cent by re-testing Limulus-negative secretions from patients with gonorrhoea at a dilution of 1 in 50, combined with the lower mean protein concentration found in secretions from such patients suggests that variation in sampling may be the main reason for the poorer results in women. This variation could possibly be overcome by standardizing the test on the basis of a determined protein concentration, for example, rather than testing all specimens at a fixed dilution. A microdilution method has also been reported to improve the sensitivity of the Limulus

assay (Prior and Spagna, 1980).

Positive Limulus results were considered to be unrelated to gonococcal infection in three (7.3 per cent) of 41 patients. It is possible that these patients may have had a gonococcal infection in the recent past of which we were unaware. However, the Limulus assay was negative in all eight patients treated for gonorrhoea within the preceding three months (less than three weeks in four patients), suggesting that gonococcal components reactive in the assay are eliminated from the cervix in a fairly short time.

Clearly, the significance of the Limulus assay requires further evaluation on a larger series of women. In particular, it would be of value to combine these studies with quantitative aspects of the "commensal" microflora of the female genital tract, to determine the extent of interference due to cervical colonization with coliform organisms and other endotoxin-positive bacteria. In men, the situation is quite different; the urethra is normally free from heavy colonization with endotoxin-positive bacteria, and as shown by Spagna et al. (1979), the gonococcus is almost invariably the cause of a positive Limulus assay for endotoxin.

4. MICROFLORA OF CERVIX AND VAGINA OF PATIENTS WITH AND WITHOUT GONORRHOEA

The main aim of this study was to determine the numbers of gonococci which could be readily aspirated from the genital tract in order to provide a measure of the degree of sensitivity required of tests to detect gonococcal components. Also, it was hoped to assess qualitatively and quantitatively the nature of the microflora in order to see which organisms might interfere with the test results.

It was considered that culture of cervical and vaginal aspirates would reflect the microflora of these sites more accurately than would culture of swab specimens. Ross (1977) demonstrated that over 90 per cent of organisms collected on swabs will adhere so tightly that they will not be released on inoculation of culture media.

Although a few studies have attempted to define the microflora of the cervix in health and disease (Ohm and Galask, 1975; Osborne, Wright and Grubin, 1979; Spagna, Prior and Perkins, 1980), quantitative analyses of gonococcal colonization are even more limited. In one such study, Lowe and Kraus (1976) determined the numbers of N. gonorrhoeae recovered in cervico-vaginal aspirates from 52 women with gonorrhoea; the numbers were within a range of 4.0×10^2 to 1.8×10^7 c.f.u. (mean = 1.45×10^5 c.f.u.) present in the original 10 ml volume aspirates. In the present study, separate cervical and vaginal aspirates

were taken from each patient. The numbers of N. gonorrhoeae recovered in cervical aspirates varied within a range of 5.4×10^3 to 8.0×10^6 c.f.u./ml (mean = 1.02×10^6 c.f.u./ml), while the corresponding values for vaginal aspirates were 5.0×10^2 to 5.2×10^6 c.f.u./5 ml (mean = 4.2×10^5 c.f.u./5 ml). The difference between the two means is not statistically significant (t 0.992; $0.1 < P < 0.5$). These results like those of Lowe and Kraus (1976) indicate a wide range in the numbers of gonococci recoverable by irrigation of the cervix and vagina.

The mean viable counts for N. gonorrhoeae in cervical aspirates were compared between patients with a healthy appearance of the cervix (mean = 3.6×10^5 c.f.u./ml) and patients with cervicitis (mean = 1.5×10^6 c.f.u./ml), as determined by clinical examination. The difference between the two means is not statistically significant (t 1.02; $0.1 < P < 0.5$). It has also been shown by Lowe and Kraus (1976) that the number of gonococci is not influenced by the use of oral contraceptives, concurrent infection with T. vaginalis and/or C. vaginale, time of douche prior to examination, or phase of the menstrual cycle at which women are examined.

The numbers of gonococci are important when considering the development of a method of detecting gonococcal components; our data suggest that any such method should be sensitive enough to detect components derived from as little as 10^2 (vaginal aspirates) to 10^3 (cervical aspirates) c.f.u. of N. gonorrhoeae. These are minimal estimates as amounts of antigen may be much

greater than numbers of organisms; non-viable gonococci would also contribute to the total content of antigen in aspirates. However, assuming that the viable counts do reflect the total amount of antigen, then there is a wide variation in amounts available for detection.

It is interesting that N. gonorrhoeae was recovered from the vaginal aspirates of 17 (85 per cent) of 20 patients with gonorrhoea. Although gonococci have been shown to adhere to vaginal epithelial cells in vitro (Forslin and Danielsson, 1980; Forslin, Danielsson and Falk, 1980), gonococci aspirated in the vaginal pool are probably derived from the cervix, the site of actual multiplication of the organisms; the mean viable count in vaginal aspirates was considerably lower than in cervical aspirates, even though the difference between the two means is not statistically significant. The general view on the establishment of gonorrhoea in women stems from the early fundamental studies by Harkness (1948) who demonstrated histopathologically that the columnar epithelial cells of the cervix were susceptible to gonococcal infection. This view was supported by electron microscopic studies of infected fallopian tubes in organ culture which confirmed that gonococci invade columnar epithelium (Ward et al., 1974).

Tables 4 and 5 illustrate the frequency of isolation of each organism from the cervix and vagina respectively of 18 patients with and 15 patients without gonorrhoea. It is appreciated that these numbers are small, and caution is required in drawing conclusions from these results. Gram-negative organisms

that might interfere with the Limulus lysate assay were isolated from two patients with no microbiological evidence of gonorrhoea. Overall, the microflora is similar in patients with and without gonorrhoea. Staphylococci were isolated more frequently from the aspirates from patients with gonorrhoea; among the organisms which were only isolated from patients with no microbiological evidence of gonorrhoea were haemolytic streptococci, E. coli, C. albicans, and T. vaginalis.

Resistance to gonococcal infection has been associated with certain components of the microflora of the genito-urinary tract (Kraus, 1980). The possibility that other microorganisms inhibit N. gonorrhoeae was first suggested by clinical cultures used in the diagnosis of gonorrhoea (Martin et al., 1967). Gonococcal colonies on contaminated cultures are also more likely to be non-viable (Shtibel, 1976). For example, N. gonorrhoeae is isolated less frequently if the culture is contaminated with C. albicans (Hipp et al., 1974; Hipp et al., 1975). In vivo antigonococcal interference has also been demonstrated in guinea pig subcutaneous chambers (Kraus et al., 1976).

Various mechanisms have been postulated to explain anti-gonococcal microbial interference. Competition for nutrients does not appear to be a major factor because replenishment of medium nutrients, or the overlay of a thin layer of fresh medium fails to alter the patterns of gonococcal inhibition (Kraus, 1980). Production and release of substances toxic to the gonococcus has been demonstrated. Lipids with antigonococcal and anti-meningococcal activity are produced by gonococci themselves

(Walstad, Reitz and Sparling, 1974). Many other microorganisms contain esterases and lipases that could liberate toxic lipids from non-toxic substrates. Chemical characterization of these lipids identified them as long-chain free fatty acids and monoacyl phosphatidylethanolamine (Walstad et al., 1974). Another group of antigonococcal compounds are bacteriocins released by Ps. aeruginosa; bacteriocins typically inhibit only bacteria of the same or closely related species, but this appears to be an exception. Moreover, a staphylococcal strain of phage type 2 has been described that contains a large virulence plasmid with genes that code for both exfoliative toxin and a specific staphylococcin with bactericidal activity against gonococci (Morris, Lawson and Rogolsky, 1978); the only Gram-negative organisms found to be susceptible to this bacteriocin were the Neisseria. This further confirms that Neisseria does not possess a typical Gram-negative cell wall as evidenced by its unusual susceptibility to penicillin and the lack of lipoprotein linking peptidoglycan with the outer membrane (Hebeler and Young, 1976a).

More recently, the properties of a somewhat different gonococcal inhibitor with bacteriostatic activity, produced by a strain of E. coli, have been described (Simpson and Davis, 1979). This inhibitor was dissimilar to previously reported gonococcal inhibitors of bacterial origin: it was stable to extremes of heat, cold and pH; it was not volatile or susceptible to proteolytic enzymes, lysozyme, lipase, DNAase, RNAase or certain chelating agents. Its activity was completely blocked

by ferric ammonium citrate. The availability of iron has been shown to influence the virulence of N. gonorrhoeae (Payne and Finkelstein, 1975). Hafiz, McEntegart and Jephcott (1977) demonstrated that iron can cause N. gonorrhoeae to revert to virulent colony-types in vitro. Competition for iron between E. coli, N. gonorrhoeae, and host cells could influence the onset and perhaps the outcome of gonorrhoea. Consequently, if the gonococcal inhibitor, which apparently binds iron, is produced by E. coli in vivo, it may play an important role in inhibiting gonococcal multiplication.

The ability of aerobic and facultatively anaerobic components of the endocervical microflora to inhibit the growth of N. gonorrhoeae in vitro was determined by an agar overlay assay (Saigh et al., 1978). Results revealed the most active inhibitors to be streptococci, staphylococci, and lactobacilli, in that order.

Among the patients with gonorrhoea, one patient was culture-positive on the second set of tests only; of the organisms isolated at the time of the first clinical examination, two (S. albus and lactobacilli) could be considered as potential gonococcal inhibitors, as determined by other investigators. Either or both of these organisms were also isolated from the vaginal aspirates of two patients with negative vaginal cultures.

Among the 17 patients with no microbiological evidence of gonorrhoea who were investigated bacteriologically, three were contacts of known cases of gonorrhoea. It would be tempting to speculate that N. gonorrhoeae was not successful in establishing

an infection because of the interfering resident microflora of the cervix and vagina. Organisms isolated from these patients included S. albus, haemolytic streptococci group B, lactobacilli, and T. vaginalis. Unfortunately, an agar overlay assay of the type described by Saigh et al. (1978) was not performed to determine whether the isolates in the present study were inhibitory to gonococci.

5. DETECTION OF GONOCOCCAL COMPONENTS IN THE CERVICAL AND
VAGINAL SECRETIONS INVESTIGATED BACTERIOLOGICALLY

Transformation assay

The sensitivity of the assay was improved markedly with the use of cervical secretions instead of swab specimens; the detection rate was increased from 33.3 per cent to 65 per cent. The proportion of clinical isolates auxotrophic for proline was not assessed; the preliminary results obtained in this respect indicate that not more than 10 per cent of false-negative results were due to proline auxotrophy of the donor strains. Moreover, gonococci were present in sufficient numbers to give a positive transformation assay; it is likely that the uneven distribution of gonococci in the aliquots, due to the presence of mucus, is also responsible for a proportion of the false-negative results obtained.

Although gonococci were present in sufficient numbers in the vaginal aspirates of 17 patients with gonorrhoea, the detection rate was only 35 per cent. Moreover, in the aspirate from patient no. 33 the viable count for N. gonorrhoeae was the lowest (5.0×10^2 c.f.u./5 ml) and still a positive transformation assay was obtained. It is possible that the aspirate also contained non-viable gonococci which would have contributed to the total gonococcal DNA content of the specimen. Another possible explanation for this discrepancy would be the variation in the distribution of gonococci in individual aliquots

of the original aspirates. Although the secretions were thoroughly mixed by vortexing at full speed prior to aliquoting, this may not have been sufficient to obtain an equal distribution of the organisms in suspension, owing to the presence of mucus in the aspirates.

In patient no. 24, N. gonorrhoeae was isolated from the cervical aspirate only, and a positive transformation result was obtained with both cervical and vaginal aspirates; it may be that N. gonorrhoeae was present in very small numbers in the vagina and had remained undetected by culture. In patient no. 26, cervical and vaginal aspirates gave a positive transformation result, yet gonococcal cultures were negative at the time of the quantitative examination; cultures became positive when re-tested one week later, although the patient denied sexual intercourse between the two clinical examinations.

Among those patients with no microbiological evidence of gonorrhoea, the cervical and vaginal aspirates were positive in patient 28 who was a contact of a known case of gonorrhoea; only the cervical aspirate was positive in one other patient (patient no. 5). Trichomoniasis was diagnosed in both patients.

Indirect sandwich ELISA

The results show that gonococcal antigens in cervical aspirates can be detected by the indirect sandwich ELISA system; 55 and 5 per cent of the cervical and vaginal secretions respectively from women with gonorrhoea gave a positive ELISA

result. From the earlier pilot experiments the minimum number of gonococci detectable was 6.6×10^3 c.f.u. (or 2.1×10^4 c.f.u./ml); 65 and 15 per cent of cervical and vaginal aspirates respectively had numbers of gonococci above this and a slightly higher positivity rate might therefore have been expected. One possible reason for this discrepancy may be the uneven distribution of gonococci in individual aliquots due to the presence of mucus in the original aspirate. The ELISA results do not correlate with the viable counts and the maximum sensitivity of the assay as determined in vitro; 5 of 7 cervical aspirates with numbers of gonococci judged insufficient to be detectable by the assay gave a positive ELISA result. This favours the view that the major antigen captured was soluble antigen and not whole cells; the ELISA result therefore depended on the availability of soluble antigen. It could be argued that non-viable gonococci also contributed to the total cell content when positive results did not correlate with viable counts. However, the false-negative results obtained when gonococci were present in sufficient numbers suggest that the availability of soluble antigen is a crucial factor in the ELISA system.

The sensitivity of the assay could be improved by using purified antigens and by affinity purification of antibody; it should then be able to detect a useful proportion of women with gonorrhoea. In these first experiments with this method, antibody to whole gonococci was used and it was not affinity-purified to isolate antigenococcal antibodies from the IgG.

Thornley et al. (1979a) have described a method of detecting gonococcal antigens by solid-phase radioimmunoassay with radioactively labelled antibody; the method was used to detect gonococcal antigens in urine sediments. A major problem in this assay was its inhibition by the supernatants of two-thirds of urine samples tested (Thornley et al., 1979b). The inhibition was reduced or completely abolished by the addition of soybean trypsin inhibitor (STI); STI-sensitive inhibition was attributed to proteolytic action on OM proteins either by proteases from pus cells or from other sources. This may also hold true for cervical and/or vaginal secretions in which proteolytic action on the OM proteins of the gonococcus could account for the decreased amount of antigen measured by the assay; it has been shown that some of these proteins are important antigenic determinants of the gonococcus (Johnston et al., 1976). As stated earlier, there was not a direct correlation between a low viable count and a negative ELISA result; this provides additional support to the view that some inhibitor(s) of the assay may be present in secretions from patients with gonorrhoea. Determination of proteolytic activity of cervical secretions would be a worthwhile part of any future investigations along these lines.

Limulus lysate assay

Following the preliminary evaluation of the Limulus lysate assay applied to cervical secretions obtained from 66 women, it was decided to combine these studies with quantitative assessments

of the commensal microflora of the female genital tract, to determine the extent of interference attributable to cervical colonization with coliform organisms and other endotoxin-positive bacteria.

While the present investigation was under way, a preliminary evaluation of the assay as a method of detecting gonococcal endotoxin in cervical exudates was reported by Spagna et al. (1980). When cervical secretions were diluted 1 in 300, positive results were obtained with 17 (94 per cent) of 18 specimens from culture-positive patients, and negative results were obtained with all of 22 specimens from culture-negative patients. Although the microflora of the cervix was defined qualitatively, neither quantitation of bacterial isolates nor cultures for Chlamydia spp. or U. urealyticum were done.

The results presented agree favourably with those of Spagna et al. (1980); a positive assay was obtained with 16 (84.2 per cent) of 19 specimens, from infected patients, when tested at a dilution of 1 in 100. When the secretions which gave a negative Limulus result were re-tested at a dilution of 1 in 50, the Limulus-positive-reactor rate was increased to 94.7 per cent, the same level as that reported by Spagna et al. (1980). It is surprising to see that exudates were used at a dilution of 1 in 300 by Spagna et al. (1980). The secretions were collected in a similar manner; 0.1 ml of cervical secretions was gently aspirated from the endocervical os with negative pressure by means of a sterile 1 ml pipette equipped with a rubber suction bulb, and the specimen was then transferred to

a plastic test tube containing 1 ml of sterile water (all material and diluent were pyrogen-free). The same Limulus reagent (Difco) was used in both studies; the sensitivity of the assay was demonstrated by the detection of 0.125 ng/ml E. coli endotoxin (Difco), and the corresponding value determined by Spagna et al. (1980) was 0.16 ng/ml. The only difference was that a 50 Test PyrotestTM vial was used in the present study, whereas Spagna et al. (1980) used single test Pyrotest vials; the latter is preferable because it involves less handling of the reagent. Moreover, according to Spagna and Prior (1981) gel formation does not appear to remain adherent to plastic surfaces, and pyrogen-free glass tubes provide the best results; pyrogen-free plastic tubes were used in our evaluation of the assay.

False-negative Limulus results did not correlate with low numbers of gonococci. One possible explanation may be the uneven distribution of gonococci in individual aliquots. Also Limulus lysates with similar sensitivities as demonstrated by extracted endotoxin standards have been shown to react differently to 'native' endotoxin in clinical specimens, and menstrual flow has an adverse effect (inhibitory) on the accuracy of the Limulus lysate assay (Spagna and Prior, 1981).

The quantitative bacteriological investigation showed that endotoxin-positive bacteria are relatively rare in cervical secretions. In spite of the presence of B. bivius and/or E. coli in aspirates from two patients with no microbiological evidence of gonorrhoea, a negative Limulus result was obtained.

As shown by Spagna et al. (1980), the sensitivity of the Limulus lysate assay for these other organisms is less than for gonococci.

Coagglutination test

All of the cervical specimens gave a negative coagglutination result. The preliminary pilot experiments indicated that the minimum number of colony forming units of N. gonorrhoeae strain 9 to give a positive reaction was 9.13×10^5 , and the corresponding value for a strain of N. meningitidis serogroup B was 2.5×10^7 .

The mean number of gonococci in cervical secretions from 19 patients was 1.02×10^6 c.f.u./ml, and the numbers were within a range of 5.4×10^3 to 8.0×10^6 c.f.u./ml. Therefore, the mean number of gonococci present in a 40 μ l test sample would be 0.4×10^5 c.f.u., approximately 23 times less than the minimum number of homologous organisms required to give a positive reaction. Even the 40 μ l test sample from the specimen with the highest viable count contained 3.2×10^5 c.f.u., or 2.9 times less than the minimum number detectable. However, soluble antigen may be important in the coagglutination test; if so, its level in cervical secretions remains insufficient to give a positive result.

GENERAL DISCUSSION

The significance of the rapid laboratory diagnosis of gonorrhoea and the limitations of conventional procedures have been discussed in detail in the General Introduction. The work embodied in this thesis represents an alternative approach to the diagnosis of gonorrhoea, based on the detection of gonococcal components in the secretions of infected patients. The problems encountered in maintaining the viability of gonococci during transport of clinical specimens may be overcome by such methods; if sufficiently rapid procedures can be devised, these could be used to expedite 'on the spot' diagnosis. The various methods investigated in this thesis have been discussed individually in the appropriate section. It is the aim of this concluding section to discuss the relative merits and shortcomings of each method, and to compare these with conventional procedures. Areas meriting further development are highlighted, and further topics of study outlined.

The only method investigated that might have merit for development as a rapid presumptive test is the Limulus lysate assay. At present, the Gram-stain is the most commonly used immediate presumptive diagnostic procedure. However, the value of Gram-staining of urethral and cervical secretions in women is limited; the 55 per cent detection rate, obtained by microscopy in the present investigation, is within the usual range of sensitivity found by Gram-staining (Barlow et al., 1976; Chipperfield and Catterall, 1976; Evans, 1976). In contrast, the Limulus lysate assay seems to offer a considerably more

sensitive alternative, detecting up to 94.7 per cent of infected patients. Also, at dilutions of secretions at which gonococci yield a positive result in the Limulus lysate assay, the presence of other endotoxin-positive bacteria does not seem to interfere with the results. A major advantage of this method is the ease with which it can be performed and the simplicity of interpreting results which make it technically undemanding. The Limulus-positive-reactor rate should be improved with the use of pyrogen-free glass tubes instead of plastic tubes, as recommended by Spagna and Prior (1981). Also, improvements in the collection of secretions, eliminating significant variations in sampling, are much needed in order to standardize the assay.

However the Limulus test, in detecting endotoxin, cannot be held to be specific; its significance in relation to the diagnosis of gonorrhoea depends upon the premise that other endotoxin-positive organisms are unlikely to produce sufficient amounts of endotoxin at the sites sampled.

The coagglutination test may in time prove useful as a tool for the immediate diagnosis of gonorrhoea. It is easy to perform and results may be obtained within ten minutes of the collection of secretions. Unfortunately, the results obtained in this study are disappointing. This is not totally unexpected since all of the specimens contained much smaller numbers of organisms than the concentrations required to give a positive result, as determined in the pilot experiment. Although

it is expected that more antigen would be available for the reaction than indicated by the viable counts, an adequate amount for a positive reaction was not present in cervical secretions. Treatment of secretions in such a way as to obtain more soluble antigen may, in the future, improve the sensitivity of the test.

Although the transformation assay and the indirect sandwich ELISA cannot be used for the immediate diagnosis of gonorrhoea, these approaches could be important in overcoming problems associated with maintaining the viability of N. gonorrhoeae.

The transformation assay using swab specimens fails to reach the expected sensitivity, as indicated by the preliminary studies. In contrast, 75 per cent of infected patients can be detected when aspirates are used instead of swab specimens. The assay, with auxotrophy as a marker, has important technical drawbacks: the preparation of defined minimal media and the procedure itself are too time-consuming, laborious, and technically demanding for routine diagnostic work. We disagree with Janik et al. (1976) who state that genetic transformation is a simple, rapid and sensitive method of detecting gonococci in clinical specimens. The transformation assay with a temperature-sensitive mutant as indicator strain (Zubrzycki and Weinberger, 1980) seems to offer a more attractive alternative to the use of an auxotrophic indicator strain, and this modification deserves further evaluation.

With the indirect sandwich ELISA, 60 per cent of infected patients can be detected. As there is no direct correlation

between the numbers of gonococci present in aspirates and the assay results, these probably depend on the availability of soluble antigen. It appears that in 45 per cent of aspirates, soluble antigen fails to reach a level high enough to give a positive ELISA result. It is also possible that inhibitors such as those reported by Thornley et al. (1979b) in urine supernatants are also present in secretions. The assay deserves further evaluation with aspirates treated in such a manner as to contain more soluble antigen, and/or treated with soybean trypsin inhibitor (STI) in the hope of eliminating the activity of STI-sensitive proteolytic enzymes which may affect the cell surface protein antigens. It may also be worthwhile to treat the secretions with DNAase and RNAase, since nucleic acids released in to the specimens because of cell lysis may interfere with the reaction. Finally, the sensitivity of the indirect sandwich ELISA may also be improved by using purified antigen(s) to immunize the rabbits and mice, and by affinity purification of antigenococcal antibody.

Although aspirates are conveniently assayable by the various methods investigated and they present the advantage of providing specimens in which the microbial content can be assessed qualitatively and quantitatively, they do present one major disadvantage. The presence of mucus in the majority of aspirates makes it impossible to obtain an even distribution of the organisms in individual aliquots of the original specimen. Also, a method of collecting secretions should be developed to

make the use of such specimens on a routine basis more convenient from the point of view of the clinician.

In spite of the fact that there was not invariably a correlation between numbers of gonococci and test results, cervical aspirates are considered more suitable than vaginal aspirates. In both the transformation assay and ELISA, more positive results are obtained with cervical aspirates. Also the lower numbers of non-gonococcal organisms in the cervix makes the latter a better site of investigation than the vagina for the detection of gonococcal components.

A major disadvantage of all of the above methods is that gonococci are not available for antibiotic sensitivity testing. This has become increasingly important with the discovery and increasing incidence of beta-lactamase producing strains of N. gonorrhoeae. Perhaps some rapid method of detecting gonococcal beta-lactamase in secretions could also be developed and this would be complementary to the approach. Another limitation of these methods in comparison with culture is related to diagnosis of infection in particular anatomical sites such as the rectum and pharynx. In spite of these limitations, it is considered that the development of methods to detect gonococcal components is a worthwhile objective and could make a significant contribution to the rapid diagnosis and hence control of gonococcal infection. Of the methods examined, the Limulus lysate assay and an ELISA system are considered to be two approaches that are particularly worthy of further investigation.

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APPENDIX A

Poster presented at the Workshop on the Genetics and Immunobiology of Pathogenic Neisseria, held in Hemavan, Sweden, June 1980.

IDENTIFICATION OF NEISSERIA GONORRHOEAE BY GENETIC TRANSFORMATION

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Journal of Medical Microbiology
13(2) pp 291-296

Preparation of transforming DNA

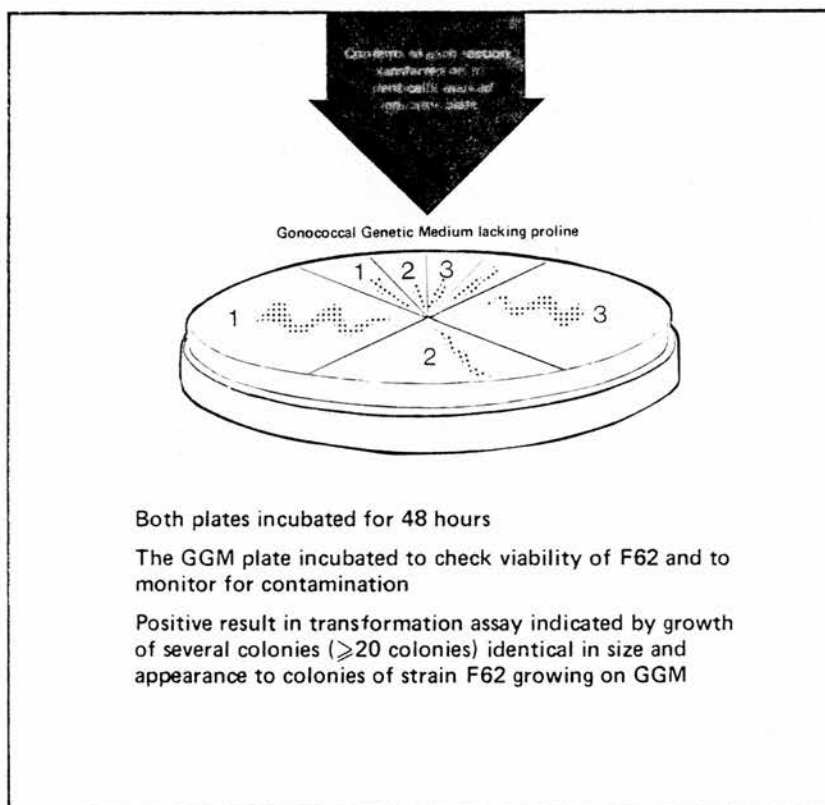
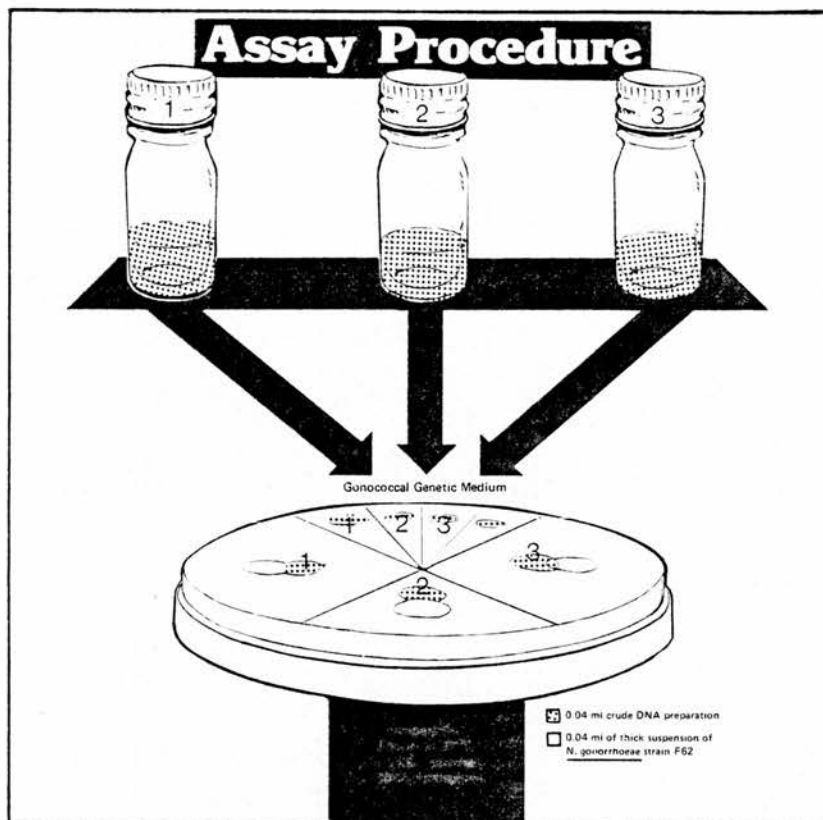
Swab charged with bacteria placed in 0.5 ml of 0.025% (w/v) sodium dodecyl sulphate in standard saline citrate solution.



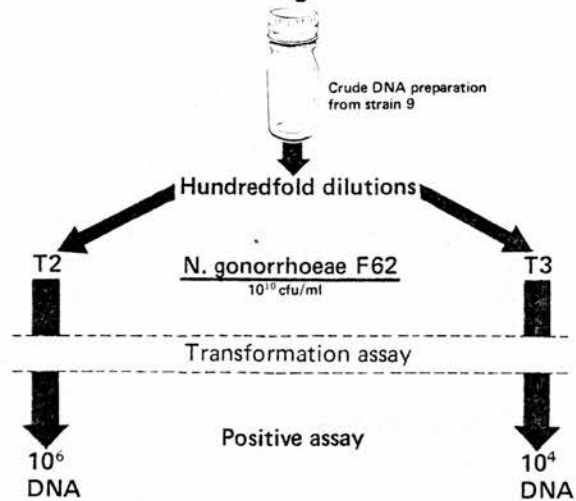
Crude DNA preparation



Water bath at 65 - 68°C
for 45 - 60 minutes



Colony-type of F62-recipient in relation to efficiency of transformation by strain 9-donor.



Colony-type 2 is more efficient than colony-type 3 in the uptake of DNA

Specificity of Assay

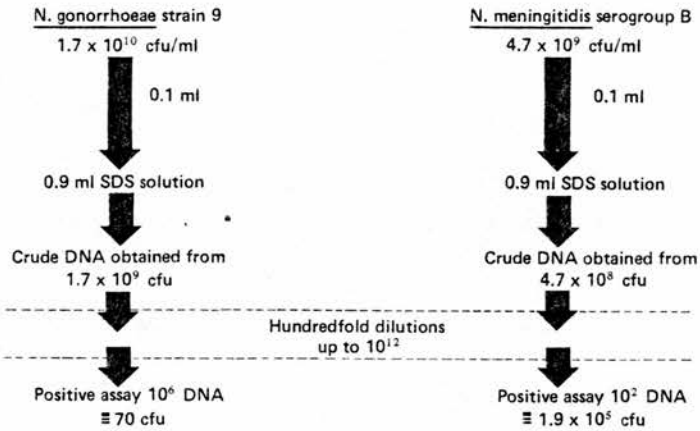
Positive transformation with:

- N. gonorrhoeae strain 9
- N. meningitidis strains of serogroups A, B, C, D, E, 29E, W135, X and Z

Negative transformation with:

- | | |
|----------------------------------|----------------------------------|
| – <u>N. elongata</u> | – <u>N. pharyngis</u> var siccus |
| – <u>N. cinerea</u> | – <u>N. denitrificans</u> |
| – <u>N. catarrhalis</u> | – <u>N. animalis</u> |
| – <u>N. cuniculi</u> | – Oxford staphylococcus |
| – <u>N. canis</u> | – <u>Pseudomonas aeruginosa</u> |
| – <u>N. pharyngis</u> var flavus | |

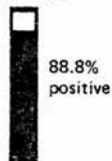
Comparative Sensitivity of Assay



The number of meningococci required to give positive transformation assay is approximately 1000-fold higher than when gonococci are used.

Clinical Isolates

N. gonorrhoeae
 n = 169



N. meningitidis
 n = 12



N. lactamica 3/3 positive
N. perflava 1/1 negative

Proportion of Clinical Isolates Auxotrophic for Proline

Of 84 isolates of gonococci tested, 73 gave a positive transformation assay.

Pattern of growth on defined media		Growth	Transformation
GGM	GGM Pro ⁺		
+	+	18	18
+	-	8	2
-	+	0	0
-	-	58	53

Requirement for proline may be main reason for negative results

APPENDIX B

Publications

Reactivity of the limulus lysate assay with uterine cervical secretions

A preliminary evaluation

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SUMMARY A limulus lysate assay was performed on cervical secretions from 66 women. When secretions were tested at a 1/100 dilution the assay gave a positive result in 15 (62.5%) of 24 patients with gonorrhoea confirmed by Gram-stained smear or culture or both. When secretions from seven of the nine remaining patients who had gonorrhoea but negative results to the limulus lysate test were retested at a 1/50 dilution, two gave a positive result, increasing the positivity rate of the test to 17 (70.8%) of 24 infected patients. Material from one patient with a history of contact with gonorrhoea and from three (7.3%) of the other 41 patients without any history of gonorrhoea gave positive reactions.

Introduction

Despite improvements in the cultural diagnosis of gonorrhoea^{1,2} and the advent of several transport and growth systems,^{3,4} problems associated with maintaining the viability of the gonococcus still exist in certain areas. Gram-staining of material from the genitourinary tract is the only widely accepted non-cultural method for the diagnosis of gonorrhoea. The reliability of Gram-staining for male cases is high; an unequivocally positive or negative result for smears of urethral discharge provides an immediate differential diagnosis between gonococcal and nongonococcal urethritis in 85% of patients.⁵ However, in female cases Gram-staining of urethral and cervical material will detect only 55-65% of patients from whom *Neisseria gonorrhoeae* is subsequently isolated by culture.⁶⁻⁸

An assay for endotoxin resulted from the finding by Levin and Bang⁹ that a lysate of washed amoebocytes of the horseshoe crab (*Limulus polyphemus*) formed a gel in the presence of minute amounts of endotoxin elaborated by Gram-negative bacteria. Since the demonstration by Rice and Kasper¹⁰ of the sensitivity of the limulus endotoxin assay for components of *N gonorrhoeae*, the system

has been shown to be of value in the rapid presumptive diagnosis of gonococcal urethritis in men.^{11,12} Because of the lack of sensitivity of Gram-staining in female patients, any new non-cultural diagnostic method is of greater potential value in the diagnosis of gonorrhoea in women than in men. For these reasons we considered it worthwhile to report our preliminary evaluation of the limulus lysate assay when applied to cervical secretions.

Patients and methods

STUDY POPULATION AND DIAGNOSIS

Sixty-six women consecutively attending the department of genitourinary medicine at the Black Street Clinic, Glasgow, were investigated. Specimens were taken from the urethra and cervix for microscopical and cultural examination and from the rectum for culture only. Cultural and identification methods for *N gonorrhoeae* were as described.¹³

COLLECTION OF SECRETIONS

The cervix was cleaned with a cotton-wool swab held in sponge-holding forceps under direct vision. Secretions were collected from the endocervical canal by gentle aspiration through a sterile polythene capillary tube (chromatography column tubing, internal diameter 1.0 mm, obtained from Pharmacia Fine Chemicals, Uppsala, Sweden), one end of which had been inserted through the os to about 1 cm; the other end of the tube was attached to a 5-ml syringe

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Accepted for publication 8 September 1980

containing 1 ml pyrogen-free water. Secretions in the tubing were ejected into a pyrogen-free plastic container. The secretions were stored at -20°C until required.

LIMULUS ASSAY

Frozen specimens of cervical secretions were thawed, mixed thoroughly, and diluted in pyrogen-free water to a final dilution of 1/100; 0.1 ml of the diluted secretion was mixed in a pyrogen-free plastic container with 0.1 ml of reagent reconstituted from the 50 Test Pyrotest™ vial (Difco Laboratories, Detroit, Michigan), incubated at 37°C in a water bath for one hour and read. Results were interpreted in accordance with the manufacturer's instructions; a firm opaque gel which remained adherent to the bottom of the vial when inverted through 180° was scored as positive; the absence of a firm gel was scored as negative. Control samples with known positive and negative results were tested with each batch of assays. The sensitivity of the limulus assay was demonstrated by the detection of $0.125\text{ }\mu\text{g/l}$ Difco Pyrotoxin positive control *Escherichia coli* endotoxin.

The limulus assays were read without previous knowledge of the conventional microbiological results. After the correlation between the limulus assay results and a diagnosis of gonorrhoea had been made, certain specimens showing an apparently false-positive limulus result were retested at a dilution of 1/200 while specimens showing a false-negative result were retested at a dilution of 1/50.

PROTEIN ESTIMATION

To determine whether false-negative results could possibly be due to the secretions being very dilute, the protein concentration of several secretions (four specimens with false-negative and five with confirmed positive results chosen at random) was determined.¹⁴

STATISTICAL ANALYSIS

The χ^2 test with Yates's correction was used to test the correlation between limulus assay results and conventional microbiological findings. Student's *t*

test was used to compare the mean protein concentrations in secretions giving false-negative and confirmed positive limulus results.

Results

CULTURE AND MICROSCOPY

The results of culture and microscopy for *N gonorrhoeae* are shown in relation to limulus lysate assay results in the table. The limulus assay gave a positive result in 19 (28.8%) of the 66 patients investigated. The result was reactive in 15 (62.5%) of the 24 patients with cervical gonorrhoea (positive smear or culture result or both) compared with only four (9.5%) of the 42 patients without any microbiological evidence of gonorrhoea. This difference is statistically highly significant (χ^2 18.4; $P < 0.001$). When secretions from seven of the nine patients with gonorrhoea but negative limulus assay results at a dilution of 1/100 were retested at 1/50 two gave a positive result and this increased the positivity rate of the test to 17 (70.8%) of 24 infected patients.

PRESENTING DIAGNOSES

The presenting diagnoses (multiple in a few cases) of the 42 patients in whom there was no microbiological evidence of gonorrhoea were as follows: *Chlamydia trachomatis* infection, eight; trichomoniasis, 10; candidosis, eight; warts, five; and no infection detected, nine. The remaining eight patients, all with negative limulus assay results, had been treated for gonorrhoea within the preceding three months (range one week to three months).

The limulus assay gave a positive result in one patient who was a contact of gonorrhoea, one patient from whom *C trachomatis* was isolated, one patient with warts, and one patient in whom no abnormality was detected. If the patient who was a contact of gonorrhoea is excluded the presumed false-positivity rate is reduced to 7.3%: on retesting secretions from two of the three remaining patients at a dilution of 1/200 both gave negative results, reducing the false-positivity rate to 2.4%.

TABLE Results of conventional microbiological investigations for *Neisseria gonorrhoeae* and of the limulus lysate assay for endotoxin in cervical secretions from 66 women

Microbiological results	No of patients	Results of limulus lysate assay	
		Positive	Negative
Culture + /smear +	15	11	4
Culture + /smear -	6	3	3
Culture - /smear +	3	1	2
Culture - /smear -	42	4	38
Total	66	19	47

+ Positive - negative

MEAN PROTEIN CONCENTRATION

The mean protein concentration in the secretions from four patients with gonorrhoea who gave negative limulus results was 0.42 g/l compared with a mean concentration of 1.50 g/l in the secretions from five patients with gonorrhoea who gave positive limulus results. The difference between the two means is statistically significant (t 10.12; $P < 0.001$).

Discussion

Although the number of infected patients was low there was a highly statistically significant correlation ($P < 0.001$) between limulus lysate assay results and conventional microbiological evidence of gonorrhoea. The limulus assay result was positive in only 62.5% of women with cervical gonorrhoea and was less sensitive than Gram-staining, which detected 75% of infected women. However, the positivity rate of 62.5% for the limulus test is within the usual range (55-65%) of sensitivity found by Gram-staining.⁶⁻⁸

In the study of Spagna *et al*¹¹ the limulus assay applied to urethral exudates from men gave a positive result in all 73 culture-positive cases of gonorrhoea tested. Our finding that the positivity rate for the limulus test could be increased to 70.8% by retesting limulus-negative secretions from patients with gonorrhoea at a dilution of 1/50, combined with the lower mean protein concentration found in secretions from such patients, suggests that variation in sampling may be the main reason for the poorer results in women. This variation could possibly be overcome by standardising the test on the basis of a determined protein concentration, for example, rather than by testing all specimens at a fixed dilution. A microdilution technique has also been reported to improve the sensitivity of the limulus assay.¹²

Positive limulus test results were considered to be unrelated to gonococcal infection in three (7.3%) of 41 patients. It is possible that these patients may have had a recent gonococcal infection, of which we were unaware. However, the limulus assay gave a negative result in all eight patients treated for gonorrhoea within the preceding three months (less than three weeks in four patients) suggesting that gonococcal components reactive in the assay are eliminated from the cervix in a fairly short time.

Clearly, the specificity of the limulus assay requires further evaluation on a larger series of women. In particular, it would be of value to combine these studies with quantitative aspects of the "commensal" flora of the female genital tract to determine the extent of interference due to cervical colonisation with coliform organisms and other endotoxin-positive bacteria. In men the situation is

quite different; the urethra is normally free from heavy colonisation with endotoxin-positive bacteria and, as shown by Spagna *et al*,¹¹ the gonococcus is almost invariably the cause of a positive limulus assay result for endotoxin.

Recently these workers¹⁵ extended their studies on the limulus assay to detect gonococcal endotoxin in cervical exudates diluted 1/800. The assay result was reactive in 17 (94%) of 18 of infected women, all of whom had a purulent cervical discharge. The lack of such a discharge in most of our infected patients might explain the lower reactivity rate in our study. Spagna *et al*¹⁵ noted that four of eight patients with nongonococcal cervicitis, in whom other Gram-negative bacteria were present, gave false-positive limulus assay results when exudates were tested at a 1/200 dilution: all eight results were negative when exudates were tested at a 1/800 dilution. Our combined findings suggest that it may prove difficult to obtain reliable results when exudates from all patients, irrespective of the presence or absence of a purulent cervical discharge, are tested at the same dilution.

This work was supported in part by a grant from the Scottish Home and Health Department (research grant No K/MRS/50/C22). We thank Professor J G Collee and Dr D H H Robertson for their helpful advice in the preparation of this paper.

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